PCT WORLD INTELLECTI Inte

INTERNATIONAL APPLICATION PUBLISHED

WO 9603506A2

(51) International Patent Classification 6 :
C12N 15/31, 1/21, 15/70, C07K 14/285, 19/00, A61K 39/102, G01N 33/569 // (C12N 1/21, C12R 1:19)

A2

(11) International Publication Number: WO 96/03506

(43) International Publication Date: 8 February 1996 (08.02.96)

(21) International Application Number: PCT/CA95/00434

(22) International Filing Date: 21 July 1995 (21.07.95)

(30) Priority Data:

08/278,091	21 July 1994 (21.07.94)	US
08/296,149	26 August 1994 (26.08.94)	US
08/487,167	7 June 1995 (07.06.95)	US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/487,167 (CIP)
Filed on 7 June 1995 (07.06.95)

(71) Applicant *(for all designated States except US)*: CONNAUGHT LABORATORIES LIMITED [CA/CA]; 1755 Steeles Avenue West, Willowdale, Ontario M2R 3T4 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LOOSMORE, Sheena, M. [CA/CA]; 70 Crawford Rose Drive, Aurora, Ontario L4G 4R4 (CA). YANG, Yan-Ping [CN/CA]; Apartment 1709, 120 Torresdale Avenue, Willowdale, Ontario M2R 3N7 (CA). CHONG, Pele [CA/CA]; 32 Estoril Street, Richmond Hill, Ontario L4C 0B6 (CA). OOMEN, Raymond, P. [CA/CA]; R.R. No. 1, Schomberg, Ontario L0G 1T0 (CA). KLEIN, Michel, H. [CA/CA]; 16 Munro Boulevard, Willowdale, Ontario M2P 1B9 (CA).

(74) Agent: STEWART, Michael, I.; Sim & McBurney, Suite 701, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ANALOG OF HAEMOPHILUS HIN47 WITH REDUCED PROTEASE ACTIVITY

(57) Abstract

The invention concerns isolated and purified analogs of *Haemophilus influenza* Hin47 protein with decreased protease activity (of less than 10 % of that of the natural protein) but preferably retaining substantially the same immunogenic properties as natural Hin47. Preferred analogs have mutations at Ser197, His91 and/or Asp121 positions and are possibly used as chimeric proteins with other immunogenic molecules. Also disclosed are nucleic acid encoding said analogs, recombinant plasmids and transformed host cells containing said modified genes, immunogenic compositions containing Hin47 analogs or their nucleic acid and their use for prophylactic, vaccine or diagnostic purposes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Title of the Invention

Analog of *Haemophilus* Hin47 with Reduced Protease Activity

Field of the Invention

The present invention relates to the field of immunology and is particularly concerned with immunogens and antigens from species of *Haemophilus*.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of United States patent application Serial No. 08/296,149 filed August 26, 1994, which itself is a continuation-in-part of Serial No. 08/278,091 filed July 21, 1994.

Background to the Invention

Haemophilus influenzae is the organism responsible for a variety of serious human diseases, such as meningitis, epiglotitis, pneumonia and otitis media. *Haemophilus influenzae* type b (Hib) is a major cause of bacterial meningitis in children under the age of five years. Protective antibodies to the disease are induced by the capsular polysaccharide of the organism and vaccines have been developed that utilise the purified polyribosyl ribitol phosphate (PRP) as the antigen. This vaccine provides 90% protection in adults and in children over 24 months of age, but was ineffective in children under 24 months (Zangwill et al 1993). (The references are identified in a list of references at the end of this disclosure, each of which reference in the list is hereby incorporated by reference without further reference thereto). Like other polysaccharide antigens, PRP does not induce the proliferation of T-helper cells, and re-immunisation fails to elicit either a booster response or an increase in memory cells. Conjugation of the PRP polysaccharide with protein carriers confers T-cell dependent characteristics to the vaccine and substantially enhances the immunologic response to the

PRP antigen. Currently, there are four PRP-carrier conjugate vaccines available. These are vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid, tetanus toxoid, or *Neisseria meningitidis* outer membrane protein (reviewed in Zangwill et al, 1993). These *H. influenzae b* conjugate vaccines have dramatically reduced the incidence of bacterial meningitis (Schoendorf et al, 1994).

There are six serotypes of *H. influenzae* designated a to f, which are defined by their capsular polysaccharides. The current *Haemophilus* conjugate vaccines do not protect against other invasive typable strains (types a and c) and, importantly, do not protect against non-typable (NTHi) strains which are a common cause of postpartum and neonatal sepsis, pneumonia and otitis media. Otitis media is the most common illness of early childhood with approximately 70% of all children suffering at least one bout of otitis media before the age of seven. Chronic otitis media can lead to hearing, speech, and cognitive impairment in children. It is caused by bacterial infection with *Streptococcus pneumoniae* (approximately 50%), non-typable *H. influenzae* (approximately 30%), and *Moraxella (Branhamella) catarrhalis* (approximately 20%). In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. To achieve universal protection against *H. influenzae* related diseases, particularly in the two to six month age group and certain high risk groups, the provision of conserved, cross-reactive non-capsular *H. influenzae* immunogens is desirable. Non-typable strains of *H. influenzae* are also important pathogens responsible for pneumonia in the elderly and other individuals who are particularly susceptible to respiratory infections. There is thus a

need for antigens from *H. influenzae* which are useful as components in immunogenic preparations that provide protection against the many serotypes of *H. influenzae*. PCT application WO 92/10936, published July 9, 1992 and incorporated herein by reference thereto, describes a 47,000 molecular weight outer membrane protein obtained from *H. influenzae* that is reported to be an adhesin and has been termed Hin47 that is immunologically conserved between non-typable, type b and non-typed clinical isolates of *H. influenzae*. The amino acid sequence of Hin47 and the nucleotide sequence of the gene encoding Hin47 were presented at the American Society of Microbiology (ASM) conference held in New Orleans, May 26-30, 1992. These sequences have also been published in PCT application WO 94/00149, published January 6, 1994 and incorporated herein by reference thereto.

Since Hin47 is conserved among strains of *Haemophilus influenzae*, and is reported to be an adhesin, the protein has utility in diagnosis of and vaccination against disease caused by *H. influenzae* or other bacterial pathogens that produce Hin47 or a protein capable of raising antibodies specifically reactive with Hin47.

A disadvantage of Hin47 for use as an antigen in diagnosis, for the generation of anti-Hin47 antibodies useful in diagnosis and as an immunogen in vaccination is the unexpected discovery by the present applicants that Hin47 has protease activity which results in the autodigestion of Hin47 and the proteolytic degradation of other antigens mixed therewith.

It would be advantageous to provide analogs of Hin47 protein (sometimes referred to herein as mutants or derivatives) that are substantially reduced in proteolytic activity for use as antigens, immunogenic preparations including vaccines, carriers for other immunogens and the generation of diagnostic reagents.

Summary of the Invention

The present invention is directed towards the provision of analogs of *Haemophilus* Hin47 protein having reduced protease activity.

In accordance with one aspect of the invention there is provided an isolated and purified analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of natural Hin47 protein. Such Hin47 analog preferably has substantially the same immunogenic properties of natural Hin47 protein. The analog of the present invention may be produced by chemical, biochemical or genetic modification of natural Hin47.

In one embodiment of the present invention, when the analog is produced by genetic modification, at least one amino acid of the natural Hin47 contributing to protease activity may be deleted or replaced by a different amino acid to produce the reduced protease activity. Alternatively, the reduced protease activity may be achieved by inserting at least one amino acid into the natural Hin47 protein. The at least one deleted or replaced amino acid may be selected from amino acids 195 to 201 of Hin47, and specifically may be Serine-197, which may be deleted or replaced by alanine, cysteine or threonine. In addition, the at least one deleted or replaced amino acid may be His-91 and may be deleted or replaced by alanine, lysine or arginine. Further, the at least one deleted or replaced amino acid may be Asp-121 and may be deleted or replaced by alanine.

In addition, multiple amino acids in the Hin47 molecule may be deleted or replaced. Such multiple amino acids may include His-91 and Serine-197 and may be deleted or replaced by Ala-91 and Ala-197 to produce a Hin47 analogue H91A/S197A. In addition, the multiple amino acids may include His-91, Asp-121 and Ser-197 and may be deleted or replaced with Ala-91, Ala-121 and Ala-

197 respectively to produce a Hin47 analogue H91A/D121A/S197A. A summary of some of the properties of some Hin47 analogues as provided herein is shown in Table 3. Only one Hin47 mutant D121E was found to retain substantial protease activity.

In a further aspect, the present invention provides an isolated and purified nucleic acid molecule comprising a mutant *Haemophilus influenzae hin*47 gene encoding an analog of *Haemophilus influenzae* Hin47 protein having a reduced protease activity which is less than about 10% of natural Hin47 protein. The mutant *hin*47 gene may encode any of the Hin47 analogs discussed above. The mutant gene preferably is formed by site-directed mutagenesis of a wild-type *hin*47 gene. The nucleic acid molecule may be contained in a recombinant plasmid adapted for transformation of a host and may be plasmid DS-1011-1-1 (deposited on July 27, 1994 at American type Culture Collection, Rockville, Maryland, U.S.A. under Accession No. 75845). The invention also includes a transformed cell containing such a recombinant plasmid.

The present invention, in another aspect, includes a method for producing an analog of *Haemophilus influenzae* Hin47 protein having a reduced protease activity which is less than about 10% of natural Hin47 protein, which comprises identifying at least one amino acid residue of Hin47 protein which contributes to protease activity thereof, effecting site-directed mutagenesis of the *hin*47 gene to remove or replace a nucleotide sequence encoding the at least one amino acid and to produce a mutated *hin*47 gene, introducing the mutated *hin*47 gene into a cell to produce a transformed cell and growing the transformed cell to produce the Hin47 analog. The at least one amino acid which is selected may be any of the ones specifically identified above with respect to the Hin47 analog.

The introduction of the mutated *hin*47 gene preferably produces a transformed cell in which the mutated *hin*47 gene is under control of the T7 promoter and the growing of the transformed cell and expression of the Hin47 analog by the T7 promoter then preferably is effected by culturing in an inducing concentration of lactose. Preferably, the introduction of the mutated *hin*47 is effected by transforming the cell with the recombinant plasmid DS-1011-1-1, sometimes otherwise referred to as plasmid pT7/Hin47*.

A further aspect of the invention provides a method of providing isolated and purified Hin47 analog, which comprises effecting the procedure described above for the production of the Hin47 analog to produce grown transformed cells harbouring inclusion bodies containing the Hin47 analog, disrupting the grown transformed cells to produce supernatant and the inclusion bodies, solubilizing the inclusion bodies to produce a solution containing Hin47 analog, chromatographically purifying the Hin47 analog from the solution free from cell debris, and isolating the purified Hin47 analog.

The analogs of Hin47 provided herein with their decreased proteolytic activity are useful as antigens in immunogenic composition, carriers for other immunogens, diagnostic agents and in the generation of diagnostic agents. The nucleic acid molecules also are useful as probes for diagnostic use and also as in immunogenic compositions.

In a further aspect of the invention, there is provided an immunogenic composition comprising an immunoeffective amount of the Hin47 analog or of the nucleic acid molecule including the gene encoding the Hin47 analog. The immunogenic composition may be formulated as a vaccine for *in vivo* administration to a host, including a human, to confer protection against diseases caused by a bacterial pathogen that produces Hin47 or a protein

capable of inducing antibodies in the host specifically reactive with Hin47. The bacterial pathogen may be a *Haemophilus* species, such as *Haemophilus influenzae*. The immunogenic compositions of the invention may further comprise at least one other immunogenic or immunostimulating material, such as an adjuvant. In an additional embodiment, the nucleic acid molecule comprising a gene encoding the Hin47 analog may be contained within a live vector, such as a pox virus, *Salmonella*, poliovirus, adenovirus, vaccinia or BCG.

The invention also extends to a method of generating an immune response in a host, including a human, comprising administering thereto an immuno-effective amount of the immunogenic compositions provided herein.

As mentioned above, the Hin47 analog provided herein is useful in diagnostic applications. Accordingly, in an additional aspect of the invention, there is provided a method of determining the presence of antibodies specifically reactive with Hin47 in a sample, comprising the steps of:

(a) contacting the sample with the Hin47 analog having substantially the same immunogenic properties as the natural Hin47 protein as provided herein to produce complexes comprising the Hin47 analog and any such antibodies present in the sample specifically reactive therewith; and

(b) determining production of the complexes.

The present invention also provides a method of determining the presence of Hin47 in a sample, comprising the steps of:

(a) immunizing a subject with an immunogenic composition as provided herein to produce antibodies specific for Hin47 protein;

(b) contacting the sample with the antibodies to produce complexes comprising any Hin47 present in the sample and the Hin47 specific antibodies; and

(c) determining production of the complexes.

The invention also extends to a diagnostic kit for determining the presence of antibodies in a sample specifically reactive with Hin47, comprising:

(a) the Hin47 analog having substantially the same immunogenic properties as the natural Hin47 protein as provided herein;

(b) means for contacting the analog with the sample to produce a complex comprising the analog and any such antibodies present in the sample; and

(c) means for determining production of the complex.

Brief Description of the Drawings

Figure 1 shows the restriction maps of plasmids JB-1031-1-14 and JB-1068-2-2 and the construction of the plasmids for sequence analysis;

Figure 2 shows the full nucleotide (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of Hin47 from *H. influenzae* strain SB33 as well as a partial nucleotide sequence (SEQ ID NO: 3) and a partial deduced amino acid sequence (SEQ ID NO: 4) thereof, the latter being specifically copied by an inventor herein from materials presented in the ASM conference as described above;

Figure 3 shows a comparison of the amino acid sequences of *H. influenzae* Hin47 (SEQ ID NO:2), *E. coli* htrA (SEQ ID NO: 5), and *Salmonella typhimurium* htrA (SEQ ID NO:6);

Figure 4 shows an alignment of amino acid residues 57 to 256 of Hin47 with certain known proteases (SEQ ID NOS: 7 to 16). Codes are as follows: TON, rat tonin; PKAAB, kallikrein; PTN, trypsin; CHAA, chymotrypsin; EST, elastase: RP2A, rat mast cell protease; SGT, Streptomyces griseus trypsin; SGBE, *S. griseus* proteinase A; SGA, S.griseus proteinase B; ALP, L.enzymogenes alpha-lytic protease; hin47, res. 57-256 of Hin47. Asterisks(*) denote structurally conserved regions. The catalytic triad residues are indicated by a hash mark (#). 'con'

refers to regions of structural concensus, among the mammalian proteases;

Figure 5 shows the restriction maps for plasmids DS-1011-1-1 and DS-1048-2 which express a Hin47 analog from *E. coli* and a construction scheme for plasmid DS-1011-1-1 (plasmid pT7/Hin47*);

Figure 6 shows a process for purifying the Hin47 analog from *E. coli* according to one embodiment of the present invention and gel analysis of the purified product;

Figure 7 shows the protease activities of natural Hin47 and Hin47 analog towards β-casein;

Figure 8 shows the stability of natural Hin47 and the Hin47 analog at different temperatures;

Figure 9 shows the enzymatic degradation of an *H. influenzae* recombinant protein by natural Hin47 and the Hin47 analog; and

Figure 10 shows the comparative immunogenicity of natural Hin47 and the Hin47 analog in mice;

Figure 11 shows the amino acid comparison of Hin47 protein isolated from *H. influenzae* strains SB33 and SB12; and

Figure 12 shows the purification of the Hin47 analogue H91A from *E. coli*.

General Description of Invention

Any *Haemophilus* strains that have Hin47 genes may be conveniently used to provide the purified and isolated nucleic acid molecules (which may be in the form of DNA molecules), comprising at least a portion coding for Hin47 as typified by embodiments of the present invention. Such strains are generally available from clinical sources and from bacterial culture collections, such as the American Type Culture collection. Such strains include *H. influenzae* strains and other bacteria that produce a protein capable of generating antibodies that

specifically recognize Hin47 fragment or analog thereof. Appropriate strains of *Haemophilus* may include:-

H. influenzae type b strain MinnA;

H. influenzae type b strain Eagan;

H. influenzae non-typable strain SB33;

H. influenzae non-typable strain SB12; or

H. influenzae non-typable strain PAK 12085.

Referring to Figure 1, there is illustrated restriction maps of plasmids JB-1031-1-14 and JB-1068-2-2 that contain a portion encoding Hin47 protein from non-typable *H. influenzae* SB33. The nucleotide sequence of the Hin47 gene was determined and is shown in Figure 2 along with the deduced amino acid sequence of the Hin47 protein. Referring to Figure 3, there is shown an amino acid sequence alignment of *H. influenzae* Hin47 and the serine proteases *htr*A from *Escherichia coli* and *htr*A from *Salmonella typhimurium*. This alignment for the first time reveals the unexpected discovery of the present applicants that Hin47 is related to bacterial serine proteases and that Hin47 has protease activity. Hin47 has previously been reported to be an adhesin. The discovered protease activity thereof greatly limits the usefulness of natural Hin47 as an immunogen for vaccination and as an antigen in diagnostic uses. The sequence alignment shown in Figure 3 revealed that the htrA proteins and Hin47 contain a GNSGGAL (SEQ ID NO: 17) sequence between residues 195 and 201 of the mature protein. The consensus sequence of the active site of serine proteases is GDSGGPK (SEQ ID NO: 18) (Brenner, 1988) and the active residue is serine. Thus, Serine-197 in Hin47 was mutated to produce an analog of Hin47 reduced in protease activity, in accordance with one embodiment of the invention. In a particular embodiment, Serine-197 was replaced by alanine. Amino acid residues 57 to 256 of Hin47 were further aligned with known proteases and the

active site residues identified from the local homologies surrounding the residues of the catalytic triad (Figure 4). There is a standard numbering system for serine proteases in which the catalytic triad residues are numbered as His-57, Asp-102 and Ser-195. These correspond to residues His-91, Asp-121 and Ser-197 in the sequential numbering system. Thus, referring to Figure 4, there is shown a structure-based alignment of ten structurally determined serine proteases (SEQ ID NOS: 7 to 16) in which homologous residues are aligned primarily on the basis of similar locations in three-dimensional space. The location of many of the residues in the hydrophobic core of Hin47, as well as residues around the active site can be aligned reasonably well to identify functional amino acids of the Hin47 protease. Thus, other amino acid residues in Hin47 that contribute to protease activity of the protein include His-91 and Asp-121. In particular embodiments, His-91 may be replaced by alanine, lysine or arginine. In an additional embodiment, Asp-121 may be replaced by alanine or glutamic acid. In an additional embodiment, Serine-197 may be replaced by alanine, serine or threonine. Although the provision of an analog of Hin47 having reduced protease activity has been exemplified herein by particular amino acid substitution within Hin47 protein, the discovery of the protease activity and the methods of Hin47 expression, purification and analysis provided herein, allow for the production of other analogs having at least one other amino acid deleted or replaced or having at least one additional amino acid inserted into the Hin47 protein. In particular applications and embodiments, it may be desirable to simultaneously alter several amino acids of the Hin47 protein to particularly reduce the protease activity of Hin47. The multiple amino acids may be His-91 and Ser-197 and may be deleted or replaced by alanine. In an alternative embodiment,

the multiple amino acids may be His-91, Asp-121 and Ser-197 and may be deleted or replaced by alanine. Accordingly, the present invention provides analogs of Hin47 protein having decreased protease activity due to single or multiple amino acid deletions, replacements or additions within the Hin47 protein.

As discussed above, Hin47 shows homology with *E. coli* htrA or *S. typhimurium* htrA, both of which are stress response proteins with serine protease activity. *E. coli* htrA is inducible by growth at 43.5°C (ref. 13). We have shown that the *E. coli* htrA protein is also inducible by growth in 6% ethanol. Hin47 can also be induced by 6% ethanol and to a lesser extent by temperature reduction at 43.5°C as described in detail below. This analysis of the expression of Hin47 provides further evidence of the relatedness between this protein and LtrA.

The *hin47* gene was also cloned from the non-typable *H. influenzae* strain SB12 by PCR amplification. Referring to Figure 11, there is shown an amino acid compariosn between the Hin47 proteins of *H. influenzae* strains SB12 and SB33. This shows the proteins to be almost identical in amino acid sequence.

Referring to Figure 5, there is illustrated plasmids DS-1011-1-1 and DS-1048-2 which express a Hin47 analog serine-197 → alanine in *E. coli*. Figure 6 shows a flow diagram of a method for the purification of the Hin47 analog from *E. coli* inclusion bodies.

Figure 7 shows the reduced protease activity of the Hin47 serine-197 → alanine analog on the substrate β-casein and demonstrates the analog to have less than about 10% of the protease activity of natural Hin47 protein. Thus, in one embodiment of the invention, there is provided an analog of Hin47 having a protease activity of less than about 10% of the protease activity of natural Hin47 and such analog may specifically have amino acid Serine-197 replaced by alanine.

Referring to Figure 8, there is illustrated an analysis of the increased stability of an analog of Hin47 as provided herein. Thus, in one embodiment of the present invention, there is provided an analog of Hin47 protein having increased thermal stability, and such analog may specifically have amino acid serine-197 replaced by alanine.

Referring to Figure 9, there is illustrated the proteolytic degradation of a non-Hin47 *Haemophilus* antigen by Hin47 and a Hin47 analog as provided herein. Thus, in accordance with a further embodiment of the present invention, there is provided an analog of Hin47 compatible with a second non-Hin47 protein and such analog may specifically have amino acid Serine-197 replaced by alanine.

Referring to Figure 10 and Table 1, there is illustrated the comparative immunogenicity of unmodified Hin47 and a Hin47 analog having reduced protease activity in mice. The Hin47 protein and Hin47 analogs S197A and H91A had comparable immunogenicity. Thus, in a particular embodiment, there is provided an analog of Hin47 having reduced protease activity and having substantially the same immunogenic properties of natural Hin47 protein. Such analog may specifically have amino acid Serine-197 replaced by alanine.

Referring to Tables 2 and 3, there is shown the immunoprotective properties of analogs of Hin47 having reduced protease activity against Hib in the infant rat model of bacteraemia and in the active immunization chinchilla model of otitis media according to particular embodiments of the invention, such analog may specifically have amino acid His-91 deleted or replaced by alanine, lysine or arsinine; Asp-121 deleted or replaced by alanine or glutamic acid; Serine-197 replaced by alanine, cysteine or threonine; or combination thereof.

In accordance with another aspect of the present invention, there is provided a vaccine against *Haemophilus* or other bacterial pathogens that produce Hin47 or a protein capable of inducing antibodies that specifically recognize Hin47, comprising an immunogenically-effective amount of an immunoprotective analog of Hin47 as provided herein or a nucleic acid molecule having a sequence encoding a Hin47 analog as provided herein, and a physiologically-acceptable carrier therefor. The provided analogs also may be used as a carrier protein for hapten, polysaccharides or peptides to make a conjugate vaccine against antigenic determinants unrelated to Hin47.

As will be apparent from the following disclosure, the present invention further provides plasmids and novel strains of bacteria for production of Hin47 analogs as provided herein.

The purified and isolated DNA molecules comprising at least a portion coding for an analog of *Haemophilus influenzae* Hin47 protein having reduced protease activity compared to natural Hin47 typified by the embodiments described herein, are advantageous as nucleic acid probes for the specific identification of *Haemophilus* strains *in vitro* or *in vivo*. The Hin47 analogs encoded by the DNA molecules provided herein are useful as diagnostic reagents as antigens or for the generation of anti-Hin47 antibodies, antigens for the vaccination against the diseases caused by species of *Haemophilus* and other bacterial pathogens that produce a protein capable of producing antibodies that specifically recognise Hin47 and for detecting infection by *Haemophilus* and other such bacteria.

In additional embodiments of the present invention, the Hin47 analogs having reduced protease activity as provided herein may be used as carrier molecules to prepare chimeric molecules and conjugate vaccines

(including glycoconjugates) against pathogenic bacteria, including encapsulated bacteria. Thus, for example, glycoconjugates of the present inventions may be applied to vaccinations to confer protection against disease and infection caused by any bacteria having polysaccharide antigens, including lipooligosaccharides (LOS) and PRP. Bacterial pathogens may include, for example, *Haemophilus influenzae, Streptococcus pneumoniae, Escherichia coli, Neisseria meningitidis, Salmonella typhi, Streptococcus mutans, Cryptococcus neoformans, Klebsiella, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Particular antigens which can be conjugated to analogs of Hin47 and methods to achieve such conjugations are described in applicants published PCT application WO 94/12641 which is hereby incorporated by reference thereto.

In another embodiment, the carrier function of Hin47 analogs may be used, for example, to induce immunity toward abnormal polysaccharides of tumor cells, or to produce anti-tumor antibodies that can be conjugated to chemotherapeutic or bioactive agents.

Accordingly, the present invention provides the primary sequence and the preparation of analogs of Hin47 of *H. influenzae* that can be used in the prevention and diagnosis of diseases caused by *H. influenzae*. In particular, the inventors discovered that the Hin47 analogs can elicit protective immune responses against live *H. influenzae* type b bacterial challenge. Thus, the present inventions have utility in vaccines. The invention also discloses the nucleotide sequences of the genes encoding the Hin47 analogs. These DNA segments may be used to provide an immunogen essentially free from other *H. influenzae* antigens, such as PRP and lipooligosaccharides (LOS), through the application of recombinant DNA technology. The Hin47 analog protein, may be produced in a suitable expression system, such as *E. coli, Haemophilus, Bacillus, Bordetella* Fungi, Yeast, Baculovirus, Poxvirus, vaccinia or mammalian expression systems. The

present disclosure further provides novel techniques which can be employed for preparing essentially pure Hin47 analogs.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, treatment of, for example, *Haemophilus* infections, and infections with other bacterial pathogens that produce proteins capable of producing antibodies that specifically recognize Hin47 and the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from Hin47 analogs as disclosed herein. The vaccine elicits an immune response in a subject which produces antibodies, including anti-Hin47 antibodies and antibodies that are opsonizing or bactericidal. Should the vaccinated subject be challenged by *Haemophilus* or other bacteria that produce proteins capable of producing antibodies that specifically recognize Hin47, the antibodies bind to and inactivate the bacterium. Furthermore, opsonizing or bactericidal anti-Hin47 antibodies may also provide protection by alternative mechanisms.

Immunogenic compositions including vaccines may be prepared as injectables, as liquid solutions or emulsions. The Hin47 analogs may be mixed with pharmaceutically acceptable excipients which are compatible with the Hin47 analog. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Methods of achieving adjuvant effect include the use of agents

such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 1 to 95% of the Hin47 analogs. The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the Hin47 analogs. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations.

The dosage may also depend on the route of administration and will vary according to the size of the host.

The concentration of antigen in an immunogenic composition according to the invention is in general about 1 to 95%. A vaccine which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

The nucleic acid molecules encoding the Hin47 analog of the present invention may also be used directly for immunization by administration of the DNA directly, for example, by injection for genetic immunization or by constructing a live vector, such as *Salmonella*, BCG, adenovirus, poxvirus, vaccinia or poliovirus. A discussion of some live vectors that have been used to carry heterologous antigens to the immune system are discussed in, for example, O'Hagan (1992). Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulmer et al, 1993.

2. Immunoassays

The Hin47 analogs of the present invention are useful as immunogens for the generation of anti-Hin47 antibodies, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of anti-bacterial, *Haemophilus*, and anti-Hin47 antibodies. In ELISA assays, the Hin47 analogs, are immobilized onto a selected surface, for example, a surface capable of binding proteins such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed

Hin47 analogs, a nonspecific protein such as a solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from 2 to 4 hours, at temperatures such as of the order of about 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test sample and the bound Hin47 analogs, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second antibody may have an associated activity such as an enzymatic activity that will generate, for example, a colour development upon incubating with an appropriate chromogenic substrate. Quantification may then be achieved by measuring the degree of colour generation using, for example, a visible spectra spectrophotometer.

3. Use of Sequences as Hybridization Probes

The nucleic acid molecules of the present invention, having the sequence of the *hin*47 analog gene, allow for the identification and cloning of the Hin47 genes from any species of *Haemophilus* and other bacteria that produce proteins capable of producing antibodies that specifically recognize Hin47.

The nucleic acid molecules having the sequence encoding the Hin47 analog of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other *hin*47 genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other *hin*47 genes. For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50° to 70°C. For some applications, less stringent hybridization conditions are required, such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results.

In a clinical diagnostic embodiment, the nucleic acid molecules encoding the *hin*47 genes of the present invention may be used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of providing a detectable signal. In some diagnostic

embodiments, an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with samples containing *hin*47 gene sequences.

The nucleic acid molecules comprising *hin*47 genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid-phase procedures, the test DNA (or RNA) from samples, such as clinical samples, including exudates, body fluids (e.g., serum, amniotic fluid, middle ear effusion, sputum, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the *hin*47 genes of the present invention under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required depending on, for example, the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

4. Expression of the Genes encoding analogs of Hin47 having reduced protease activity

Vectors perhaps containing replicon and control sequences which are derived from species compatible with the host cell may be used for the expression of the Hin47 analog genes as provided herein in expression systems. The vector ordinarily carries a replication site, as well

as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the host cell for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM™-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such as *E. coli* LE392.

Promoters commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al, 1979; Goeddel et al, 1980) and other microbial promoters, such as the T7 promoter system (U.S. Patent 4,952,496). Details concerning the nucleotide sequences of promoters are known, enabling a skilled worker to ligate them functionally with plasmid vectors. The particular promoter used will generally be a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the Hin47 analogs include *E. coli, Bacillus* species, *Haemophilus Bordetella* fungi, yeast, mammalian cells or the baculovirus expression system may be used.

Thus, in accordance with the invention, it may be preferred to make the Hin47 analog protein by recombinant methods. Particularly desirable hosts for expression in this regard include Gram positive bacteria which do not have LPS and are therefore endotoxin free. Such hosts include species of *Bacillus* and may be particularly useful for the production of non-pyrogenic Hin47 analog.

Biological Deposits

Plasmid DS-1011-1-1 (pT7/Hin47*) that contains a portion coding for a Hin47 analog that is described and referred to herein has been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland, USA, pursuant to the Budapest Treaty and prior to the filing of this continuation-in-part application on July 27, 1994 under Accession No. 75845. Samples of the deposited plasmid will become available to the public upon grant of a patent based upon this United States patent application. The invention described and claimed herein is not to be limited in scope by plasmid deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

Examples

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example illustrates the cloning of the *hin*47 gene from non-typable *H. influenzae* strain SB33.

Chromosomal DNA was prepared from *H. influenzae* strain SB33, and an EMBL3 library was prepared and screened with a labelled oligonucleotide probe specific for the 5′-end of *hin*47. Non-typable *H. influenzae* strain SB33 was grown on Mueller-Hinton agar or in brain heart infusion broth as described by Harkness et al, 1992. Chromosomal DNA was prepared as follows: cells from 50 ml of culture were pelleted by centrifugation at 5000 rpm for 15 to 20 min, at 4°C, in a Sorvall RC-3B centrifuge. The cell pellet was resuspended in 10 ml of TE (10 mM Tris/HCl, 1 mM EDTA, pH 7.5), pronase was added to 500 $\mu g\ ml^{-1}$ and SDS to 1%. The sample was incubated at 37°C until a clear lysate was obtained. The lysate was gently extracted once with Tris-saturated phenol (pH 7.4), once with Tris-saturated phenol/chloroform (1:1) and once with chloroform. The final aqueous phase was dialysed at 4°C for 24 h against 1M NaCl, followed by 24 h against TE.

An EMBL3 library was prepared by partial digestion of SB33 chromosomal DNA with *Sau*3A I, followed by size fractionation either on a 10 to 30% sucrose gradient in TNE (20 mM Tris/HCl, 5 mM NaCl, 1 mM EDTA, pH 8.0) or by preparative gel electrophoresis. Fractions containing DNA fragments greater than 5 kb in length were pooled, precipitated and ligated with *Bam*H I arms of EMBL3 (Promega). The ligation mixture was packaged using a Gigapack II packaging kit and plated onto *E. coli* LE392 cells. The libraries were amplified and stored at 4°C in the presence of 0.3% chloroform.

Plaques were lifted onto nitrocellulose filters for hybridization with a ^{32}P-labelled oligonucleotide probe (3026.SL). The oligonucleotide sequence was ATGAAAAAAACACGTTTTGTATTAAATAGTATTGCACTTGG (SEQ ID NO: 3) corresponding to the N-terminal amino acid sequence MKKTRFVLNSIALG (SEQ ID NO: 19). Phage DNA was prepared from putative plaques and the insert DNA was excised by *Sal* I digestion and cloned into pUC8-BgXb digested with *Sal*

I. Plasmids JB-1031-1-14 and JB-1068-2-2 (Fig. 1) were selected for further analysis.

Example 2

This Example illustrates the characterization and sequence analysis of the *hin*47 gene and deduced amino acid sequence of the Hin47 protein from NTHi strain SB33.

Restriction mapping and Southern blot analysis of clones JB-1031-1-14 and JB-1068-2-2 localized the *hin*47 gene on a 4.7 kb *Bam*H I/*Bam*H I or a 2.7 kb *Bam*H I/*Pst* I DNA fragment. The 4.7 kb *Bam*H I/*Bam*H I fragment from JB-1068-2-2 was subcloned into pUC8/*Bg*Xb generating plasmid DS-755-1. The 3.1 kb *Bam*H I to *Xba* I fragment of DS-755-1 was subcloned into pUC18 generating plasmid JB-1165-1 which has restriction sites suitable for the Erase-a-base (Promega) procedure (Fig. 1). This technique generates successive clones with increasing truncations of insert DNA, with the deletions occurring from the same end. The resultant nested set of clones can be sequenced rapidly using a universal primer.

DNA from plasmid JB-1165-1 was digested with *Bam*H I and *Sac* I and subjected to exoIII digestion using an Erase-a-base kit. The resultant set of truncated plasmids was analysed by agarose gel electrophoresis and representative plasmids were selected for sequence analysis.

Plasmid DNA for sequencing was prepared by a modification of the procedure of Holmes and Quigley, 1981. Briefly, the cell pellet from 50 ml of culture was resuspended in 10 ml STET (8% sucrose, 5% Triton X-100, 50 mM EDTA, and 50 mM Tris/HCl, pH 8.0), lysozyme (2.5 mg) was added and the mixture was boiled for 2 min. The sample was spun at 14,000 rpm in a Sorvall RC 5B for 20 minutes and the supernatant was precipitated with an equal volume of isopropanol, washed with 70% ethanol then absolute ethanol, and then air dried. The pellet was resuspended in 0.9 ml of TE, then 20 μl of 5 mg ml^{-1} RNAse

A were added, and the mixture was incubated at 37°C for 15 min. After the addition of 500 µl of 1.5M NaCl/30% PEG, the mixture was incubated on ice for 30 min and the DNA was pelleted by centrifugation in an Eppendorf microfuge for 10 min. The pellet was resuspended in 400 µl of TE and extracted twice with Tris-saturated phenol (pH 7.4), twice with Tris-saturated phenol/chloroform (1:1) and twice with chloroform. The DNA was precipitated by adding 40 µl of 3M ammonium acetate and 1 ml of ethanol, washed with 70% ethanol and resuspended in distilled water.

DNA samples were sequenced using the ABI model 370A DNA sequencer and the dye terminator chemistry. The universal reverse primer was used with the nested set of clones to determine the sequence of the *hin*47 coding strand. Oligonucleotide primers of approximately 25 bases in length were used to confirm the sequence of the non-coding strand. The nucleotide sequence of the SB33 *hin*47 gene and the deduced amino acid sequence of the Hin47 protein are shown in Figure 2. The nucleotide and N-terminal amino acid sequences of Hin47 presented at the ASM meeting, New Orleans, May 26 to 30, 1992 are indicated in lower case on Figure 2. The amino terminal sequences of the SB33 Hin47 and this presented sequence are identical, establishing the identity of the cloned gene as *hin*47.

Example 3

This Example describes the discovery of serine protease activity of Hin47 protein.

The deduced amino acid sequence of Hin47 protein determined in Example 2 above was compared with all other known proteins in the Genbank data base. As described above, Hin47 protein is described in published PCT applications WO 94/00149, WO 92/11367 and WO 92/10936 to be an adhesin molecule of *Haemophilus*. It was, therefore, a surprising and unexpected discovery of the present

invention that Hin47 protein has significant amino acid homology (55%) with the serine proteases *E. coli* htrA and *S. typhimurium* htrA and other proteases. These amino acid sequence homologies are shown in Figures 3 and 4. Furthermore, Hin47 protein was found to autodigest unless it was stored in the presence of a serine protease inhibitor, such as Pefablock.

Example 4

This Example illustrates the generation of the mutant *hin*47 gene by site-directed mutagenesis.

As explained above, *H. influenzae* Hin 47, *E. coli* htrA, and *S. typhimurium* htrA are all serine proteases. The consensus sequence of the active site of serine proteases is GDSGGPK (SEQ ID NO: 18) [Brenner, 1988] with serine being the active residue. The htrA proteins both have a GNSGGAL (SEQ ID NO: 17) sequence and in *H. influenzae* Hin47, there is the identical sequence between residues 195 and 201 of the mature protein. Thus, the serine residue at position 197 was selected for site-directed mutagenesis, to produce an analog of Hin47 with reduced protease activity.

An oligonucleotide CGCTCCACCAGCATTACCGCGG (SEQ ID NO: 20) was synthesized which would change the serine residue at 197 to an alanine. The *hin*47 gene was cloned into M13mp18 generating clone DS-981-3 and mutagenesis was performed using the Amersham In Vitro Site-Directed Mutagenesis kit. Clone DS-991-8 was confirmed by sequence analysis to contain the mutation Serine-197 to Alanine. This mutant *hin*47 gene is designated *hin*47*. Using appropriate oligonucleotides, the serine residue at 197 was changed to a cysteine (mutant S197C) and a threonine (mutant S197T)

In addition a comparison of the amino acid sequence of Hin47 with other proteases (as shown in Figure 4) revealed that amino acids His-91 and Asp-121 are sites appropriate for mutagenesis to produce an analog of Hin47

with reduced protease activity. By mutagenesis methods analogous to those described above, His-91 and/or Asp-121 were deleted or replaced by different amino acids. Such amino acid replacements included His-91 to Alanine (mutant H91A) and Arginine (mutant H91R) and Asp-121 to Alanine (mutant D121A) and Glutamic acid (mutant D121E). Oligonucleotides to effect such mutagenesis included: His-91 → Ala-91 5′ ATCAATAACAGCATTATTGGT 3′ (SEQ ID NO: 21)

Asp-121 → Ala-121 5′ TAATGCAATTGCTGATAGTTC3′ (SEQ ID NO: 22).

Corresponding oligonucleotides were employed to effect the other mutations. Multiple mutations also were effected in which His-91 and serine-197 both were replaced by Alanine (mutant H91A/S197A) and His-91, Alp-121 and Ser-197 were all replaced by Alanine (mutant H91A/D121A/S197A).

These additional mutants were produced, extracted, purified and tested for protease activity as described for the Hin47* material in the succeeding examples.

Many serine proteases are secreted in an inactive ('zymogen') form, and require clipping to expose their active sites. N terminal sequence analysis of mature natural Hin47 protein suggested the cleavage of the preprotein to occur at KFFFG DRFAEQ (SEQ ID NO: 23). Modifications of amino acids that prevent cleavage of the molecule to produce the active protease molecule can produce an analog of Hin47 having reduced protease activity.

Example 5

This Example illustrates the construction of plasmids expressing Hin47 Ser-197 → alanine analog from *E. coli*.

The mutated *hin*47* gene from plasmid DS-991-8 was cloned into the pT7-7 expression vector to generate plasmid DS-1011-1-1 (Fig. 5). *E. coli* strain BL21/DE3 was

transformed to generate *E. coli* strain DS-1018-3-1 which expresses Hin47 Ser-197 → alanine analog upon induction.

In order to utilize tetracycline selection, the *hin*47* gene was cloned into pBR328. The Bgl II/Cla I T7/*hin*47* gene fragment from DS-1011-1-1 was cloned into pEVvrf1 (Young and Davis, 1985) in order to generate a *Bgl* II/*Bam*H I fragment which could be cloned into pUC-4K (Pharmacia) digested with BamH I. The resultant clone DS-1034-3 was digested with EcoR I and the T7/*hin*47* gene fragment cloned into pBR328 (Boehringer Mannheim Corporation) to generate plasmids DS-1048-2 and DS-1067-2. Electroporation of plasmid DNA into *E. coli* strain BL21/DE3 resulted in strains DS-1071-1-1 and DS-1071-3-1 which express the Hin47 Ser-197 → alanine analog.

Example 6

This Example illustrates the expression of Hin47 Ser-197 → alanine analog from *E. coli*.

An overnight culture of strains DS-1018-3-1, DS-1071-1-1, or DS-1071-3-1 were grown overnight in NZCYM media + 3% dextrose + antibiotics (ampicillin at 25μg ml^{-1} or tetracycline at 10μg ml^{-1}), at 37°C, with shaking. A 1:40 dilution of the overnight culture was inoculated into the same medium and grown at 37°C with shaking until the absorbance was A_{578} approximately 0.3. A 1/10 volume of 10% lactose was then added to induce expression from the T7 promoter. Cell samples were harvested about 4 hours after induction by centrifuging culture samples at 5000 rpm for 10 min in a Sorvall RC-3B, at 4°C.

Example 7

This Example illustrates the extraction and purification of Hin47.

Hin47 was expressed as soluble protein in *E. coli*. The cell pellet from a 250 ml culture, prepared as described in Example 6, was resuspended in 40 ml of 50 mM Tris-HCl, pH 8.0, and disrupted by sonication (3 x 10 min, 70% duty

circle). The extract was centrifuged at 20,000 x g and the resulting supernatant which contained > 95% of the soluble Hin47 protein was retained. This fraction was called "Hin47-extract".

This Hin47-extract was further purified on a DEAE Sephacel column. Forty ml of the Hin47-extract was applied onto a 20-ml DEAE Sephacel column equilibrated in 50 mM Tris-HCl, pH 8.0. Hin47 bound to the column under these conditions. The column was washed with 100 ml of 50 mM Tris-HCl, pH 8.0, and then washed with 100 ml of 50 mM Tris-HCl, pH 8.0 containing 20 mM NaCl. Hin47 was then eluted with 50 mM Tris-HCl, pH 8.0, containing 40 mM NaCl. The amount of Hin47 in the fractions was determined by the BCA protein assay. The purity of Hin47 was assessed by SDS-PAGE analysis. The fractions containing Hin47 were combined and stored at -20°C.

Only the H91A mutant was as soluble as the wild-type Hin47 protein, most of the other mutants being produced as inclusion bodies.

Example 8

This Example illustrates the extraction and purification of Hin47 Ser-197 → alanine analog.

Hin47 Ser-197 → alanine analog was expressed in inclusion bodies in *E. coli*. The cell pellet from a 250 ml culture, prepared as described in Example 6, was resuspended in 40 ml of 50 mM Tris-HCl, pH 8.0, and disrupted by sonication (3 x 10 min, 70% duty circle). The extract was centrifuged at 20,000 x g and the resulting pellet was saved. The pellet was re-extracted with 40 ml of 50 mM Tris-HCl, 0.5% Triton X-100, 10 mM EDTA, pH 8.0. The suspension was sonicated 10 min at 70% duty circle. The extract was centrifuged at 300 x g for 5 min. The resultant supernatant was centrifuged again at 20,000 x g for 30 min and the resultant pellet was saved. The pellet was resuspended in 50 mM Tris-HCl, 0.5% Triton X-100, 10 mM EDTA, pH 8.0. The suspension was then mixed

with 50 mM Tris-HCl, pH 8.0 containing 8 M urea. The final urea concentration in the mixture was adjusted to 2 M with 50 mM Tris-HCl, pH 8.0. Hin47 Ser-197 → alanine analog was completely solubilized under these conditions. The final volume of the solution was 20 ml. This fraction is called "Hin47 analog-extract". The Hin47 analog-extract was further purified on a DEAE Sephacel column. Twenty ml of Hin47 analog-extract was applied onto a 10ml DEAE Sephacel column equilibrated in 50 mM Tris-HCl, pH 8.0. Hin47 Ser-197 → alanine analog bound to the column under these conditions. The column was washed with 50 mM Tris-HCl, pH 8.0, and Hin47 analog was eluted with 50 mM Tris-HCl, pH 8.0, containing 30 mM NaCl. The amount of Hin47 analog in the fractions was determined by the BCA protein assay. The purity of Hin47 analog was assessed by SDS-PAGE analysis (Fig. 6). The fractions containing Hin47 analog were combined and stored at -20°C.

Example 9

This Example illustrates the protease activity of Hin47 and Hin47 Ser-197 → alanine analog.

The enzymatic activity of Hin47 and Hin47 Ser-197 → alanine analog was analyzed using β-casein as a substrate (Figure 7). The reaction mixtures contained 5 μg of β-casein and either Hin47 or Hin47 analog. The reaction was carried out at 37°C for two hours, and then stopped by adding the SDS-sample buffer and instantly heating the sample at 100°C for 5 min. The aliquots were analyzed by SDS-PAGE. As shown in Figure 7, digestion of β-casein by Hin47 was more obvious after two hours (panel A, lane 1) in comparison to the fractions containing Hin47 analog (panel A, lane 2) or without any exogenous proteins (panel A, lane 3). The presence of Hin47 or Hin47 analog in these mixtures were confirmed by immuno-blotting using a monoclonal antibody to Hin47 (Fig. 7, panel C, lanes 1 and 2).

The protease activities of Hin47 and Hin47 Ser-197 → alanine analog were also compared by analyzing the autodigestion of Hin47 or Hin47 analog at 4°C and -20°C. The purified Hin47 or analog were stored at either 4°C or -20°C for up to 20 days. Aliquots were taken on days 0, 10 and 20 and the stability of Hin47 or analog was analyzed by immuno-blotting using a Hin47 monoclonal antibody (Fig.8). The analog was much more stable than Hin47 up to 20 days when stored at either 4°C or -20°C.

To further examine the protease activity of the Hin47 Ser-197 → alanine analog, the ability of Hin47 or analog to degrade an 80-kDa *H. influenzae* recombinant antigen was examined. Thus, a mixed antigen study was performed to determine the proteolytic effect of Hin47 or Hin47 analog on another antigen. An 80 kDa *H. influenzae* recombinant protein (TBP1) was chosen for this study in order to distinguish it from the Hin47 or analog protein (47 kDa). Five mixtures were formulated as follows: 80-kDa protein alone; 80-kDa protein + Hin47; 80-kDa protein + analog; Hin47 alone; and analog alone. The amount of each protein in the mixture was 5 μg. The mixtures were stored at 4°C up to four weeks. Aliquots were taken on days 0, 7, 14 and 28 for analysis by SDS-PAGE (Fig. 9). Both the 80 kDa protein and Hin47 were largely degraded after one week (lanes 2 and 4). In contrast, the 80 kDa protein in combination with Hin47 analog remained intact after one week, and showed only slight degradation even after four weeks (lane 3).

The residual protease activity of other Hin47 analogues was assessed using the digestion of β-casein as described by Lipinska et al (ref. 13) and the results of which are shown in Table 3. Only one mutant (D121E) was found to retain serine protease activity.

Example 10

This Example illustrates the comparative immunogenicity of Hin47 and Hin47 analog in mice.

The results of a study to determine the comparative immunogenicity of Hin47 and the Hin47 Ser-197 → alanine analog are shown in Figure 10. Thus, groups of five Balb/c mice were injected three times (as indicated by arrows) s.c. on days 1, 29 and 43 with 1-μg dose of either Hin47 or Hin47 analog in the presence of $AlPO_4$ (1.5 mg per dose). Blood samples were taken on days 14, 28, 42 and 56 (as indicated by bleeds 1, 2, 3 and 4, respectively) for analyzing the anti-Hin47 antibody titers by EIAs. The determination of anti-Hin47 antibodies in mouse sera was performed as described by Panezutti et al. (1993). Microtiter wells were coated with 1 μg of either Hin47 or Hin47 analog for 16 hours at room temperature. The plates were then blocked with 0.1% (w/v) bovine serum albumin in PBS. The mouse sera were serially diluted, added to the wells, then incubated for one hour at room temperature. Affinity-purified $F(ab')_2$ fragments of goat anti-mouse IgG (Fc specific) antibody conjugated to horseradish peroxidase were used as the second antibody. The reactions were developed using tetramethylbenzidine (TMB/ H_2O_2) and absorbencies were measured at 450 nm (using 540 nm as a reference wavelength) in a Flow Multiskan MCC microplate reader. The reactive titer of an antiserum was defined as the reciprocal of the dilution consistently showing a two-fold increase in absorbance over that obtained with the pre-bleed serum sample. As can be seen from Figure 10, both Hin47 and the Hin47 analog elicited comparable IgG titers in mice regardless of whether Hin47 or mutant was used as an antigen in EIAs.

Immunogenicity studies were also performed using the H91A Hin47 analogue. This analogue was found to produce an immune response equivalent to that of the S197A Hin47 analogue.

To further examine the immune response to Hin47 or the Hin47 Ser-197 → alanine analog, the subclasses of

anti-Hin47 IgG in mouse sera were determined. Microtiter wells were coated with 1 μg of purified Hin47 or analog. The final bleed of mouse serum samples from the comparative immunogenicity study (as described above) were pooled and tested in EIAs. Rat anti-mouse IgG_1, IgG_{2a}, IgG_{2b} antibodies conjugated horseradish peroxidase and rabbit anti-mouse IgG_3 conjugated to horseradish peroxidase were used as reagents in EIAs. The working dilution of each conjugate was determined using purified antibody subclasses to avoid cross reactivity. The reactive titers were determined as described above. As shown in Table 1 below, the IgG-subclass profile induced in mice by either Hin47 or Hin47 analog were identical, regardless of whether Hin47 or analog was used as a solid antigen in the EIAs. The predominant IgG response in both groups of mouse sera was of the IgG_1 isotype. Hence, the Hin47 analog exhibited substantially the same immunogenic properties as the natural protein.

Example 11

This Example illustrates the immunoprotective properties of Hin47 and Hin47 Ser-197 → alanine analog.

The immunoprotective properties of Hin47 and the Hin47 Ser-197 → alanine analog were analyzed by the ability of Hin47 specific antisera to protect infant rats against *H. influenzae* type b strain MinnA in a bacteremia model. The results of this study are shown in Table 2 below. Groups of nine 6-day old Wistar infant rats were inoculated subcutaneously (s.c.) on the dorsum close to the neck with 0.1 mL of either a rabbit anti-Hin47 analog antiserum or the corresponding prebleed serum. Twenty-four hours later, the animals were challenged intraperitoneally (i.p.) with 700 cfu of freshly grown Hib strain MinnA. Blood samples were collected 20 hours post-challenge and plated onto chocolate agar plates. Bacterial colonies were counted after 24 hours. As shown in Table 2, three out of nine animals in the group

inoculated with anti-Hin47 analog antiserum did not show any bacteremia in their blood. Only one mouse in the group inoculated with anti-Hin47 analog antiserum (11%) had a higher bacteria recovery from the blood sample compared to mice inoculated with prebleed serum. In contrast, bacteria were recovered from all the nine mice inoculated with pre-bleed serum. Four out of nine animals (44%) in the group inoculated with pre-bleed serum showed high levels (500 to 1,000) of bacteria recovered in blood samples.

The infant rat model of bacteremia, was used to assess the protection afforded by anti-Hin47 or anti-Hin47 mutant antisera against bacteremia caused by *H. influenzae* type b infection. 6/10 infant rats were protected by antisera raised against each of wild-type Hin47, H91A Hin47, and S197A Hin47 analogues.

Example 12

This Example illustrates the induction of Hin47 under stress conditions.

H. influenzae strain Eagan was grown at 37°C to an A_{590} ≈ 0.3 in brain heart infusion broth (BHI) containing hemin (2μg ml^{-1}) and NAD (2μg ml^{-1}). The sample was aliquotted and grown at 37°C, 42°C, 43.5°C, or in the presence of 6% ethanol, 0.2M NaCl, or 0.3 M NaCl. *E. coli* strain JM109 was grown at 37°C to an A_{590} of ≈ 0.3 in YT media and aliquotted as described. Samples were collected at 0 min, 20 min, 40 min, 60 min, and 90 min and analyzed by OD and SDS-PGE/Western blot. Guinea pig antisera which recognized both *H. influenzae* Hin47 and *E. coli* htrA was used for Western blot analysis. The *E. coli* htrA protein was produced in large quantities when the organism was grown at 43.5°C and a small amount of the *H. influenzae* Hin47 protein can be observed. With growth in media containing 6% ethanol, both the *E. coli* htrA and the *H. influenzae* Hin47 proteins are induced. The high salt

conditions were insufficient to induce either protein. These results indicate that Hin47 is a stress response protein in *H. influenzae*, inducible under similar conditions to the *E. coli* htrA protein.

Example 13

This Example illustrates the purification of the H91A Hin47 protein.

The soluble H91A mutant was purified essentially as described for the wild-type Hin47 in Example 7, with the addition of a hydroxylapatite (HAP) column. The HAP column was equilibrated in 10 mM sodium phosphate buffer (pH 8.0) and the run-through from the DEAE column was loaded. The H91A Hin47 bound to the HAP column and contaminating proteins were removed by washing the column with 175 mM sodium phosphate buffer. The H91A Hin47 protein was eluted with 300 mM sodium phosphate buffer (pH 8.0) and stored at -20°C.

Example 14

This Example illustrates the protection studies with Hin47 and Hin47 mutants in the chinchilla model of otitis media.

The chinchilla model of otitis media (ref. 14) was used to assess the protection induced by active immunization with wild-type Hin47, H91A Hin47, or S197A Hin47.

Chinchillas (~500 g weight) were immunized i.m. three times with 30 μg/dose of Hin47 or Hin47 mutant (H91A or S197A) adjuvanted in AlPO4, on days 1, 28 and 42. The animals were challenged on day 56, through the bulla, with 50-1000 cfu of virulent NTHi strain SB12 organims. Animals were monitored by tympanometry and otoscopic examination and at 4 days post-challenge, middle ear fluids were aspirated and plated on chocolate agar. Bacterial colonies were counted after 24h. The wild-type Hin47 and H91A Hin47 proteins afforded

protection to ~50% of the animals, but the S197A Hin47 was non-protective in this model (Table 3).

Summary of Disclosure

In summary of this disclosure, the present invention provides novel analogs of *Haemophilus influenzae* Hin47 protein which have a decreased protease activity of less than about 10% of that of the natural Hin47 protein as well as isolated and purified DNA molecules encoding the same. Modifications are possible within the scope of this inventions.

TABLE 1

Hin47 IgG titers in mouse immune sera

IgG Suclass	IgG titers in Group #1		IgG titers in Group #2	
	To Hin47	To Mutant	To Hin47	To Mutant
IgG(H+L)	102,400	102,400	102,400	102,400
IgG_1	25,600	25,600	25,600	25,600
IgG_{2a}	< 100	< 100	< 100	< 100
IgG_{2b}	400	400	400	400
IgG_3	< 100	< 100	< 100	< 100

Group #1: Immune sera were pooled from a group of five mice received Hin47 immunization.

Group #2: Immune sera were pooled from a group of five mice received Hin47 mutant immunization.

Plates were coated with either Hin47 or mutant protein.

TABLE 2

Protective ability of rabbit Anti-Hin47 Mutant antiserum against Hib in infant rat model of bacteremia

Antibody	Number of Animals				Total Animals
	cfu of Bacteria/ 2.5 μL Blood				
	Av.0	Av.50 (10-100)	Av.200 (100-300)	Av.650 (300-1,000)	
Anti-Hin47*	3	3	2	1	9
Prebleed	0	4	1	4	9

Groups of nine 6-day old infant rats were immunized s.c. with either a rabbit anti-Hin47 mutant antiserum or the corresponding prebleed serum. Animals were challenged i.p. with 700 cfu *H. influenzae* strain MinnA after 24 hours. The blood samples were taken at 20 hours after the challenge.

Anti-Hin47* antibody: rabbit immune serum raised against purified Hin47 mutant in CFA/IFA.

Average bacteria recovery from immunized group: 100 cfu per 2.5 μL of blood; from control group: 290 cfu per 2.5 μL of blood.

TABLE 3

Characterization of Hin47 mutants

Mutant	Protease[a]	Solubility[b]	Protection - rat[c]	Protection - chinchilla[d]
WILD-TYPE	+	+	+	±
H91A	-	+	+	±
H91R	-	-	ND[e]	ND
D121A	-	-	ND	ND
D121E	+	-	ND	ND
S197A	-	-	+	-
S197C	-	±	ND	ND
S197T	-	±	ND	ND
H91A/S197A	-	-	ND	ND
H91A/D121A/S197A	-	-	ND	ND

a Protease activity is measured by the ability to digest the substrate β-casein.
b Solubility indicates production as a soluble protein (+) or inclusion bodies (-).
c Protection in the infant rat passive model of bacteremia.
d Protection in the chinchilla model of otitis media.
e ND is not determined

Reference List

1. Zangwill et al, 1993 MMWR 42:1-15.

2. Schoendorf et al, 1994 Pediatrics 93:663-8.

3. Brenner et al, 1988 Nature 334:528-530.

4. O'Hagan 1992 Clin. Pharmokinet. 22:1-10.

5. Ulmer et al, 1993 Curr. Opinion. Invest. Drugs 2:983-989.

6. Chang et al, 1978 Nature 275:617.

7. Goeddel et al 1980 Nucl. Acid. Res. 8:4057.

8. Harkness et al, 1992 J. Bacteriol. 174:2425-2430.

9. Loeb et al, 1987 Infec. Immun. 55:2612-2618.

10. Holmes and Quigley 1981. Analyt. Biochem. 114:193-197.

11. Young and Davis 1985 Gene 38:31-38.

12. Panezutti et al, 1993 Infec. Immun. 61:1867-72.

13. Lipinska et al, 1985 Bacteriol. 171:1574-1584.

14. Barenkamp et al, 1986 Infect. Immun. 52:572-578.

CLAIMS

What we claim is:

1. An isolated and purified analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein.

2. The analog of claim 1 having substantially the same immunogenic properties as natural Hin47 protein.

3. The analog of claim 1 wherein at least one amino acid of the natural Hin47 protein contributing to protease activity has been deleted or replaced by a different amino acid, or at least one amino acid has been inserted into the natural Hin47 protein, to provide said reduced protease activity.

4. The analog of claim 3 wherein said at least one deleted or replaced amino acid is selected from amino acids 195 to 201 of natural Hin47 protein.

5. The analog of claim 4 wherein said at least one amino acid is Serine-197.

6. The analog of claim 5 wherein Serine-197 is replaced by alanine, cysteine or threonine.

7. The analog of claim 3 wherein said at least one amino acid is Histidine-91 or Asp-121 of natural Hin47 protein.

8. The analog of claim 7 wherein Histidine-91 is replaced by alanine, lysine or arginine.

9. The analog of claim 7 wherein Asp-121 is replaced by alanine.

10. The analog of claim 4 wherein multiple amino acids are deleted or replaced.

11. The analog of claim 10 wherein the multiple amino acids are His-91 and Ser-197 and are deleted or replaced by alanine.

12. The analog of claim 10 wherein the multiple amino acids are His-91, Asp-121 and Ser-197 and are deleted or replaced by alanine.

13. An isolated and purified nucleic acid molecule comprising a mutant *Haemophilus influenzae hin*47 gene encoding an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein.

14. The nucleic acid molecule of claim 13 wherein said encoded analog has substantially the immunogenic properties of natural Hin47 protein.

15. The nucleic acid molecule of claim 13 wherein at least one codon of a wild-type *hin*47 gene encoding an amino acid contributing to protease activity has been deleted or replaced, or at least one codon has been inserted into the wild-type *hin*47 gene to form said mutant *hin*47 gene.

16. The nucleic acid molecule of claim 15 wherein the at least one deleted or replaced codon encodes at least one amino acid from amino acids 195 to 201 of natural Hin47 protein.

17. The nucleic acid molecule of claim 16 wherein the at least one codon is that encoding Serine-197.

18. The nucleic acid molecule of claim 17 wherein the codon encoding Serine-197 is replaced by a codon encoding alanine, cysteine or threonine.

19. The nucleic acid molecule of claim 15 wherein the at least one codon encodes His-91 or Asp-121 of natural Hin47 protein.

20. The nucleic acid molecule of claim 19 wherein the codon encoding His-91 is replaced by a codon encoding alanine, lysine or arginine.

21. The nucleic acid molecule of claim 19 wherein the codon encoding Asp-121 is replaced by a codon encoding alanine.

22. The nucleic acid molecule of claim 13 wherein said mutant gene is formed by site-directed mutagenesis of a wild-type *hin*47 gene.

23. The nucleic acid molecule of claim 10 wherein multiple codons are deleted or replaced.

24. The nucleic acid molecule of claim 23 wherein the multiple codons encode His-91 and Ser-197 and are deleted or replaced by codons encoding alanine.

25. The nucleic acid molecule of claim 23 wherein the multiple codons encode His-91, Asp-12 and Ser-197 and are deleted or replaced by codons encoding alanine.

26. A recombinant plasmid adapted for transformation of a host comprising a plasmid vector into which has been inserted the nucleic acid molecule of claim 13.

27. The recombinant plasmid of claim 26 which is plasmid DS-1011-1-1 (pT7/Hin47*) deposited under ATCC designation no. 75845.

28. A transformed cell containing the recombinant plasmid of claim 26.

29. A method for producing an analog of *Haemophilus influenzae* Hin47 protein having a reduced protease activity which is less than about 10% of natural Hin47 protein, which comprises:

identifying at least one amino acid residue of Hin47 protein which contributes to protease activity thereof;

effecting site-directed mutagenesis of the *hin*47 gene to remove or replace a nucleotide sequence encoding said at least one amino acid and to produce a mutated *hin*47 gene;

introducing the mutated *hin*47 gene into a cell to produce a transformed cell; and

growing the transformed cell to produce the Hin47 analog.

30. The method of claim 29 wherein said at least one amino acid is selected from amino acids 95 to 201 of natural Hin47 protein.

31. The method of claim 30 wherein said at least one amino acid is Serine-197.

32. The method of claim 30 wherein Serine-197 is replaced by alanine, cysteine or threonine.

33. The method of claim 29 wherein said at least one amino acid is Histidine-91 or Asp-121 of natural Hin47 protein.

34. The method of claim 33 wherein Histidine-91 is replaced by alanine, lysine or arginine.

35. The method of claim 33 wherein Asp-121 is replaced by alanine.

36. The method of claim 29 wherein multiple amino acids are deleted or replaced.

37. The method of claim 36 wherein the multiple amino acids are His-91 and Ser-197 and are deleted or replaced by alanine.

38. The method of claim 36 wherein the multiple amino acids are His-91, Asp-121 and Ser-197 and are deleted or replaced by alanine.

39. The method of claim 29 wherein said introduction of the mutated *hin*47 gene produces a transformed cell in which the mutated *hin*47 gene is under control of the T7 promoter, and said growing of said transformed cell and expression of the Hin47 analog by said T7 promoter is effected by culturing in an inducing concentration of lactose.

40. The method of claim 39 wherein said introduction of the mutated *hin*47 gene is effected by transforming said cell with the recombinant plasmid DS-1011-1-1 (pT7/Hin47*) deposited under ATCC designation 75845.

41. An immunogenic composition, comprising an immuno-effective amount of an analog of Hin47 as claimed in claim 2 or a nucleic acid molecule as claimed in claim 14.

42. The immunogenic composition of claim 41 formulated as a vaccine for *in vivo* administration to a host to confer protection against diseases caused by a bacterial pathogen that produces Hin47 protein or a protein capable

of inducing antibodies in the host specifically reactive with Hin47 protein.

43. The immunogenic composition of claim 42 wherein the bacterial pathogen is a *Haemophilus* species.

44. The immunogenic composition of claim 43 wherein the *Haemophilus* species is *Haemophilus influenzae*.

45. The immunogenic composition of claim 42 further comprising at least one other immunogenic or immunostimulating material.

46. A method of generating an immune response in a host comprising administering thereto an immuno-effective amount of the immunogenic composition of claim 41.

47. A method of determining the presence of antibodies specifically reactive with Hin47 protein in a sample, comprising the steps of:

(a) contacting the sample with the Hin47 analog of claim 2 to produce complexes comprising the Hin47 analog and any said antibodies present in the sample specifically reactive therewith; and

(b) determining production of the complexes.

48. A method of determining the presence of Hin47 protein in a sample comprising the steps of:

(a) immunizing a subject with the immunogenic composition of claim 41 to produce antibodies specific for Hin47 protein;

(b) contacting the sample with the antibodies to produce complexes comprising any Hin47 protein present in the sample and said Hin47 protein specific antibodies; and

(c) determining production of the complexes.

49. A diagnostic kit for determining the presence of antibodies in a sample specifically reactive with Hin47 protein, comprising:

(a) the Hin47 analog of claim 2;

(b) means for contacting the analog with the sample to produce complexes comprising the analog and any said antibodies present in the sample; and

(c) means for determining production of the complexes.

50. A method of providing isolated and purified analog *Haemophilus influenzae* Hin47 analog, which comprises:

effecting the method of claim 29 to produce grown transformed cells harbouring inclusion bodies containing the Hin47 analog;

disrupting said grown transformed cells to produce supernatant and said inclusion bodies;

solubilizing said inclusion bodies to produce a solution containing Hin47 analog;

chromatographically purifying said Hin47 analog from said solution free from cell debris; and

isolating said purified Hin47 analog.

51. The method of claim 50 wherein the method of claim 23 is effected according to the method of claim 39.

52. A chimeric molecule, comprising an analog as claimed in claim 1 linked to a polypeptide, protein or a polysaccharide.

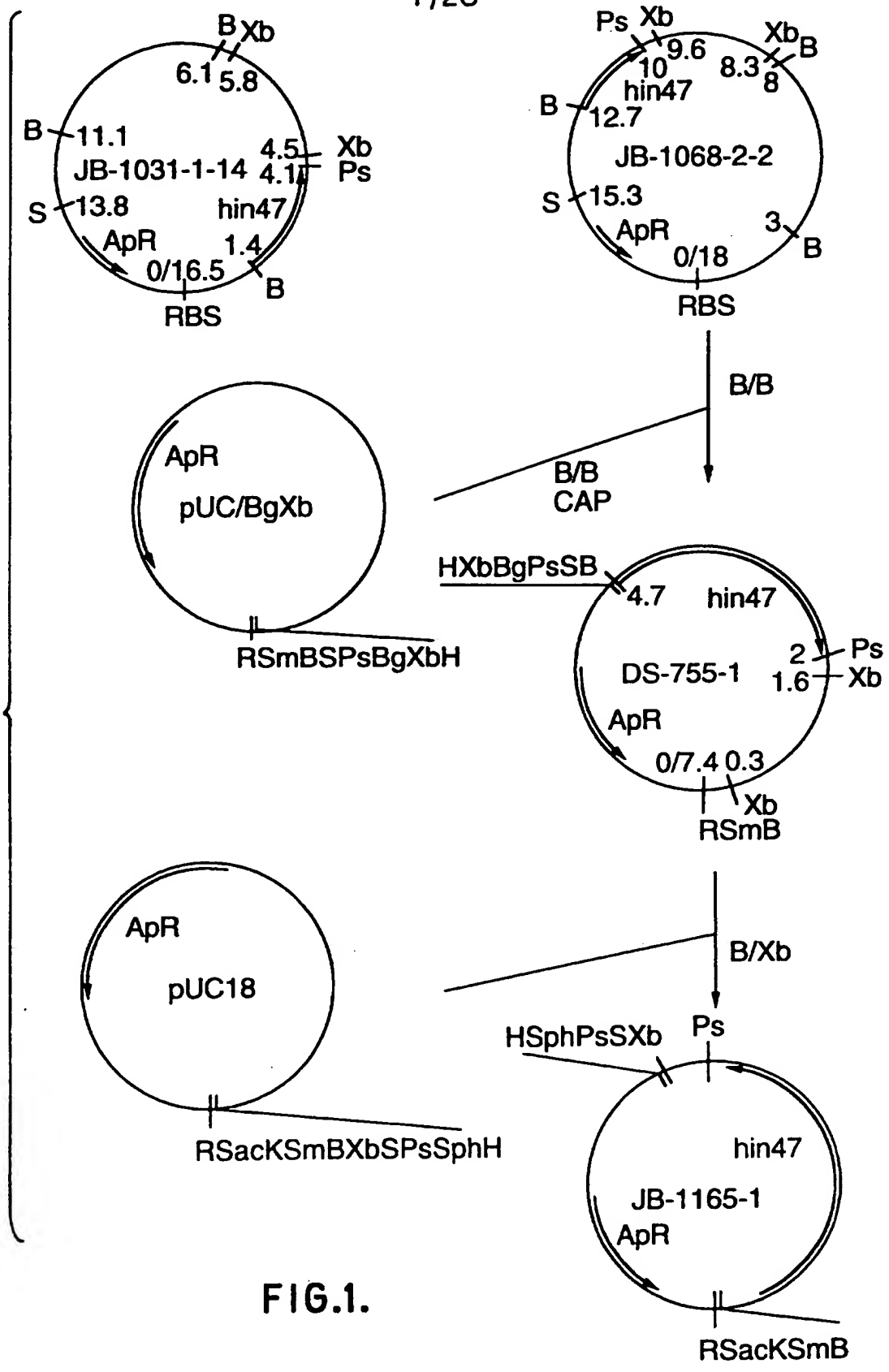


FIG.1.

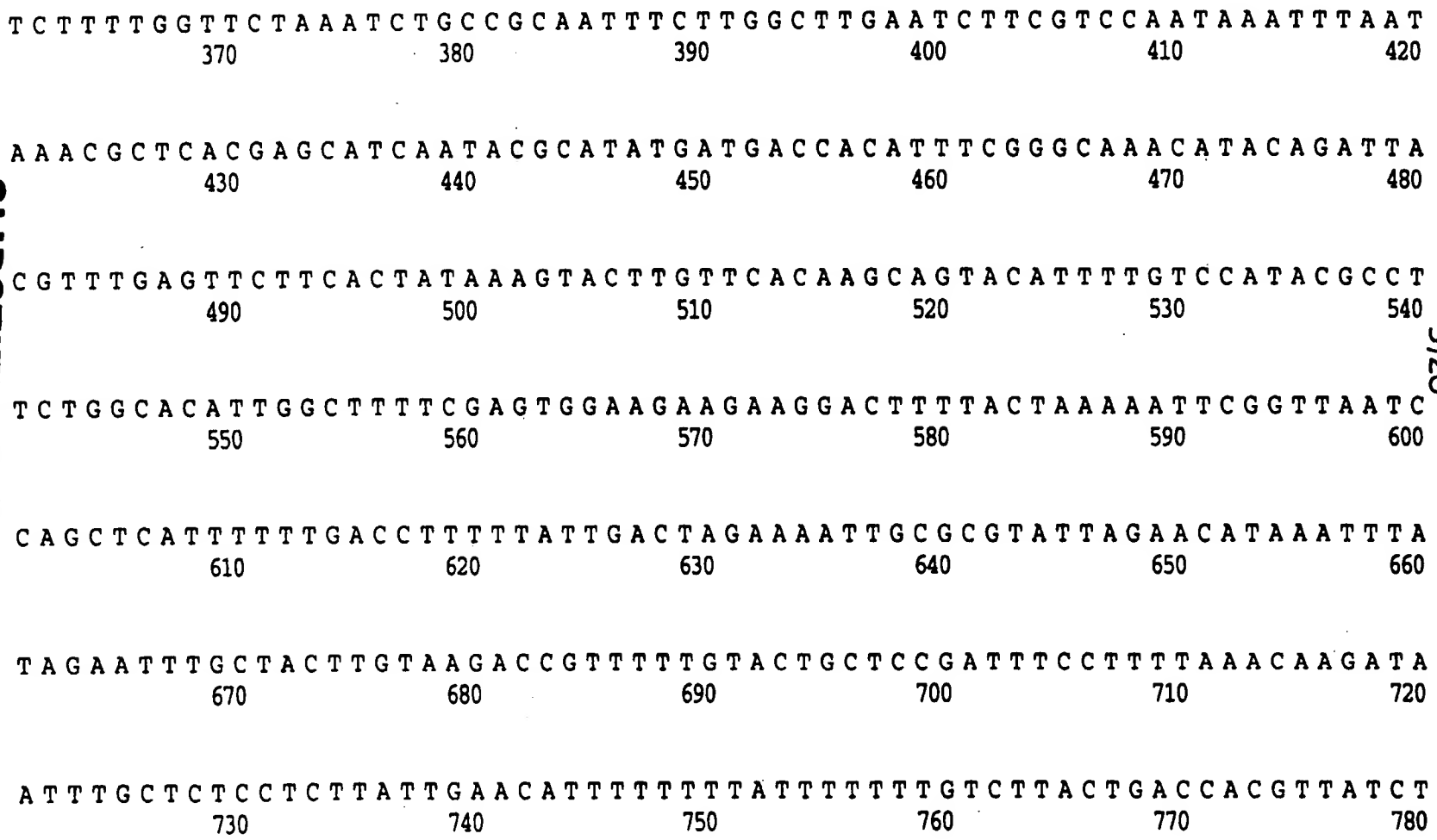
SUBSTITUTE SHEET

FIG.2A.

SB33 Hin47 sequence

```
GGATCCGTTAATACTGAAATAAATGGCACACCTTTTTCACGCATTTGGGCAAGTACAGCA
          10        20        30        40        50        60

CTGGTTTTTGCCATTTGCATTAAAGAGAATAATGCTTCCTGCATACGAGCACCACCACTC
          70        80        90       100       110       120

GCAGAGAAACATACAAACGGACAATTCATTTCCATCGCTTTTTCAGCCGCTTTAACAAAT
         130       140       150       160       170       180

TTTGCACCAACTACAGAACCCATTGAACCGCCCATAAAAGCAAAGTTCGATGCAGCCACA
         190       200       210       220       230       240

ACAATTGGCATATCATAAAGTGTACCTGTCATAGTAATTAGCGCATCTTTCTCGCCCGTT
         250       260       270       280       290       300

TCTTTTTGTGCCGCATTGATACGATCTTTATATTTCTTTAAATCTTTAAATTTTAAAATA
         310       320       330       340       350       360
```

FIG.2B.



SUBSTITUTE SHEET

FIG.2C.

```
                                    met  lys  lys  thr  arg  phe  val  leu  asn  ser  ile  ala  leu
                                    MET  LYS  LYS  THR  ARG  PHE  VAL  LEU  ASN  SER  ILE  ALA  LEU
G A A A T T T A T T T T G G A G T A T T T A T G A A A A A A A A C A C G T T T T G T A C T A A A T A G T A T T G C A C T T
                                    a t g a a a a a a a a a c a c g t t t t g t a t t a a a t a g t a t t g c a c t t
          790                 800                 810                 820                 830                 840

gly  leu  ser  val  leu  ser  thr  ser  phe  val  ala  gln  ala  thr  leu  pro  ser  phe  val  ser
GLY  LEU  SER  VAL  LEU  SER  THR  SER  PHE  VAL  ALA  GLN  ALA  THR  LEU  PRO  SER  PHE  VAL  SER
G G A T T A A G T G T A T T A A G C A C A T C A T T T G T T G C T C A A G C C A C T T T G C C A A G T T T T G T T T C G
g
          850                 860                 870                 880                 890                 900

glu  gln  asn  ser
GLU  GLN  ASN  SER  LEU  ALA  PRO  MET  LEU  GLU  LYS  VAL  GLN  PRO  ALA  VAL  VAL  THR  LEU  SER
G A A C A A A A A C A G T C T T G C A C C A A T G T T A G A A A A A G T A C A A C C T G C C G T T G T C A C T C T T T C C
          910                 920                 930                 940                 950                 960

VAL  GLU  GLY  LYS  ALA  LYS  VAL  ASP  SER  ARG  SER  PRO  PHE  LEU  ASP  ASP  ILE  PRO  GLU  GLU
C T T G A A G G A A A A G C T A A A G T A G A T T C T C G T T C T C C T T T C C T A G A C G A T A T T C C T G A A G A A
          970                 980                 990                 1000                1010                1020

PHE  LYS  PHE  PHE  PHE  GLY  ASP  ARG  PHE  ALA  GLU  GLN  PHE  GLY  GLY  ARG  GLY  GLU  SER  LYS
T T T A A A T T C T T C T T T G G C G A T C G T T T T G C C G A A C A A T T T G G T G G A C G T G G A G A A T C A A A G
          1030                1040                1050                1060                1070                1080
```

SUBSTITUTE SHEET

FIG.2D.

```
ARG  ASN  PHE  ARG  GLY  LEU  GLY  SER  GLY  VAL  ILE  ILE  ASN  ALA  SER  LYS  GLY  TYR  VAL  LEU
CGTAACTTCCGTGGTTTAGGTTCTGGTGTCATTATTAATGCAAGCAAAGGCTATGTTTTA
          1090              1100              1110              1120              1130              1140

THR  ASN  ASN  HIS  VAL  ILE  ASP  GLU  ALA  ASP  LYS  ILE  THR  VAL  GLN  LEU  GLN  ASP  GLY  ARG
ACCAATAATCATGTTATTGATGAAGCTGATAAAATTACCGTGCAATTACAAGATGGGCGT
          1150              1160              1170              1180              1190              1200

GLU  PHE  LYS  ALA  LYS  LEU  VAL  GLY  LYS  ASP  GLU  LEU  SER  ASP  ILE  ALA  LEU  VAL  GLN  LEU
GAATTTAAAGCAAAATTAGTGGGTAAAGATGAACTATCAGATATTGCATTAGTACAGCTT
          1210              1220              1230              1240              1250              1260

GLU  LYS  PRO  SER  ASN  LEU  THR  GLU  ILE  LYS  PHE  ALA  ASP  SER  ASP  LYS  LEU  ARG  VAL  GLY
GAAAAACCAAGTAATTTAACAGAAATCAAATTTGCTGATTCCGACAAATTACGCGTAGGC
          1270              1280              1290              1300              1310              1320

ASP  PHE  THR  VAL  ALA  ILE  GLY  ASN  PRO  PHE  GLY  LEU  GLY  GLN  THR  VAL  THR  SER  GLY  ILE
GATTTCACTGTTGCAATCGGTAATCCATTTGGTTTAGGTCAAACTGTGACATCAGGTATT
          1330              1340              1350              1360              1370              1380

VAL  SER  ALA  LEU  GLY  ARG  SER  THR  GLY  SER  ASP  SER  GLY  THR  TYR  GLU  ASN  TYR  ILE  GLN
GTTTCTGCATTGGGTCGTTCAACAGGTTCTGACAGTGGCACTTATGAAAACTATATTCAA
          1390              1400              1410              1420              1430              1440

THR  ASP  ALA  ALA  VAL  ASN  ARG  GLY  ASN  SER  GLY  GLY  ALA  LEU  VAL  ASN  LEU  ASN  GLY  GLU
ACCGATGCAGCAGTAAACCGCGGTAATTCGGGTGGAGCGTTAGTAAACTTAAATGGCGAA
          1450              1460              1470              1480              1490              1500
```

FIG.2E.

```
 LEU  ILE  GLY  ILE  ASN  THR  ALA  ILE  ILE  SER  PRO  SER  GLY  GLY  ASN  ALA  GLY  ILE  ALA  PHE
CTTATTGGAATTAATACCGCAATTATTTCTCCAAGCGGTGGCAATGCAGGAATTGCCTTT
          1510          1520          1530          1540          1550          1560

 ALA  ILE  PRO  SER  ASN  GLN  ALA  SER  ASN  LEU  VAL  GLN  GLN  ILE  LEU  GLU  PHE  GLY  GLN  VAL
GCGATTCCAAGTAATCAAGCAAGCAATTTAGTGCAACAAATTTTAGAATTTGGTCAAGTG
          1570          1580          1590          1600          1610          1620

 ARG  ARG  GLY  LEU  LEU  GLY  ILE  LYS  GLY  GLY  GLU  LEU  ASN  ALA  ASP  LEU  ALA  LYS  ALA  PHE
CGTCGCGGATTGCTTGGTATTAAAGGTGGCGAACTCAATGCTGATTTAGCCAAAGCCTTT
          1630          1640          1650          1660          1670          1680

 ASN  VAL  SER  ALA  GLN  GLN  GLY  ALA  PHE  VAL  SER  GLU  VAL  LEU  PRO  LYS  SER  ALA  ALA  GLU
AATGTAAGCGCGCAACAAGGCGCATTTGTAAGTGAAGTTTTACCGAAATCTGCTGCTGAA
          1690          1700          1710          1720          1730          1740

 LYS  ALA  GLY  LEU  LYS  ALA  GLY  ASP  ILE  ILE  THR  ALA  MET  ASN  GLY  GLN  LYS  ILE  SER  SER
AAAGCAGGACTTAAAGCGGGCGATATTATCACGGCGATGAACGGTCAAAAAATCTCAAGT
          1750          1760          1770          1780          1790          1800

 PHE  ALA  GLU  ILE  ARG  ALA  LYS  ILE  ALA  THR  THR  GLY  ALA  GLY  LYS  GLU  ILE  SER  LEU  THR
TTCGCTGAAATTCGTGCAAAAATCGCAACCACTGGTGCAGGCAAAGAGATTAGCTTGACT
          1810          1820          1830          1840          1850          1860
```

FIG.2F.

```
 TYR  LEU  ARG  ASP  GLY  LYS  SER  HIS  ASP  VAL  LYS  MET  LYS  LEU  GLN  ALA  ASP  ASP  SER  SER
TACTTACGTGATGGCAAATCCCACGACGTTAAAATGAAATTACAAGCGGATGATAGTAGC
           1870         1880         1890         1900         1910         1920

 GLN  LEU  SER  SER  LYS  THR  GLU  LEU  PRO  ALA  LEU  ASP  GLY  ALA  THR  LEU  LYS  ASP  TYR  ASP
CAACTTTCCTCAAAAACTGAGTTGCCTGCATTAGATGGTGCAACATTGAAAGACTACGAT
           1930         1940         1950         1960         1970         1980

 ALA  LYS  GLY  VAL  LYS  GLY  ILE  GLU  ILE  THR  LYS  ILE  GLN  PRO  ASN  SER  LEU  ALA  ALA  GLN
GCTAAAGGCGTTAAAGGAATTGAAATCACAAAAATTCAACCTAATTCGCTGGCTGCACAA
           1990         2000         2010         2020         2030         2040

 ARG  GLY  LEU  LYS  SER  GLY  ASP  ILE  ILE  ILE  GLY  ILE  ASN  ARG  GLN  MET  ILE  GLU  ASN  ILE
CGTGGTTTAAAATCGGGCGATATTATTATTGGTATTAATCGTCAAATGATCGAAAACATT
           2050         2060         2070         2080         2090         2100

 ARG  GLU  LEU  ASN  LYS  VAL  LEU  GLU  THR  GLU  PRO  SER  ALA  VAL  ALA  LEU  ASN  ILE  LEU  ARG
CGTGAATTAAATAAAGTGCTTGAAACTGAACCGTCAGCAGTTGCACTTAATATTTTACGA
           2110         2120         2130         2140         2150         2160

 GLY  ASP  SER  ASN  PHE  TYR  LEU  LEU  VAL  GLN  ***
GGTGACAGTAATTTCTATTTATTAGTGCAATAATCTGCTTGATATATTTAAGAAAAAAGT
           2170         2180         2190         2200         2210         2220
```

SUBSTITUTE SHEET

FIG.2G.

```
CCGATCACAATGATCGGGCTTCTTTTTATGCAGCAATCGTTCTTAACAAATCCACCACAA
          2230      2240      2250      2260      2270      2280

ATTCTAACCGCACTTTGTTATCAGATAAATCTTTCATGAACTTAAATTTTAATGGGCCAT
          2290      2300      2310      2320      2330      2340

CAAATCGATACACAATAGGTTCTTTTTGAATTAATTGAATAAATTTATCTGGATTCACTT
          2350      2360      2370      2380      2390      2400

GTGCTTTTGCTGAAAACTCAATAAAACCGCCTTGTGTTCCTGCATCAATTCGCACAACTT
          2410      2420      2430      2440      2450      2460

TCAACGGCTCAACCAACAAACGCAATTCTGCAATTTGCAGTAAATTTTTTGTTGCATCAG
          2470      2480      2490      2500      2510      2520

GCAATAATCCGAATCGATCTATTAACTCAACTTTTAATTCATCTAATTCTGCTTTACTCT
          2530      2540      2550      2560      2570      2580

CTGCTGCAGCAATGCGTTTATAAAAGGATAAACGCATATTCACGTCTCCTAGATAATCAT
          2590      2600      2610      2620      2630      2640
```

FIG.2H.

```
CAGGCAGTAAAGCAGGCACACGCAATTCAATATCCGCTTGTTGTTGCGTCAATTCTTCTA
2650      2660      2670      2680      2690      2700

ATGATGGTTCACGCCCTTCTTTTAACGCTTTAACCGCTGCATCCAATAATTCCATATAAA
2710      2720      2730      2740      2750      2760

CGAAAAACCGATGCTTTCAATTTGTCCACTTTGTTCCAAGTAATTCGCCGGCACC
2770      2780      2790      2800      2810      2820

GACGAATCTCTAAATCGTGGGTTGCCAAGATAAACCAGCCCCAAGATTATCAAGATTTT
2830      2840      2850      2860      2870      2880

CAACGCATCTAGA
2890
```

FIG.3A.

Comparison of Hin47 with E.coli htrA and S.typhimurium htrA

```
MKKTRFVLNSIALGLS---VLS-TSFVAQATLPSFVSEQ--NSLAPMLEKVQP
....TLA.SRL..S..--LA..PL.AT.AE.-S.ATTA.QMP.........M.
....--T.AMS..A..LGLA..PL.AT.AE.SS.AMTA.QMP.........M.

                                AVVTLSVEGKAKV-DSRSP------FLDDIP--EEFKFFFGDRF--A  Hin47
                                S..SIN...STT.NTP.M.RNFQQF.G..S.FCQ.GSP.QSSP.CQG  E. coli
                                S..SIN...STT.NTP.M.RNFQQF.G..S.FCQDGSP.QNSP.CQG  S. typh

                                          _
EQFGGRGESKRNFRGLGSGVIINASKGYVLTNNHVIDEADKITVQLQDGREFK
G.G.NG.GQQQK.MA.......D.D....V.....V.N.TV.K...S...K.D
GGN.GN.GQQQK.MA.......D.A....V.....V.N.SV.K...S...K.D

                                          _
                                AKLVGKDELSDIALVQLEKPSNLTEIKFADSDKLRVGDFTVAIGNPF  Hin47
                                ..M....PR.....I.IQN.K...A..M....A.....Y..G.....  E. coli
                                ..V....PR.....I.IQN.K...A..L....A.....Y........  S.typh

                                     _
GLGQIVTSGIVSALGRSTGSDSGTYENYIQTDAAVNRGNSGGALVNLNGELIG
...E.............-.LNAEN...F......I..................
...E.............-.LNVEN...F......I..................
```

FIG. 3B.

```
INTAIISPSGGNAGIAFAIPSNQASNLVQQILEFGQVRRGLLGIKGG  Hin47
.....LA.D...I..G.....MVK..TS.MV.Y...K..E...M.T  E. coli
.....LA.D...I..G.....MVK..TS.MV.Y.....E...M.T  S. typh

ELNADLAKAFNVSAQQGAFVSEVLPKSAAEKAGLKAGDIITAMNGQKISSFAE
...SE....MK.D..R.....Q...N.S.A...I....V..SL..KP.....A
...SE....MK.D..R.....Q.M.N.S.A...I....V..SL..KP.....A

                        IRAKIAITGAGKEISLITYLRDGKSHDVKMKLQADDSSQLSSKTELPA  Hin47
                        L..QVG.MPV.SKLT.GL.....QVN.NLE...QSSQN.VD.SSIFNG  E. coli
                        L..QVG.MPV.SK...GL..E..ATT.NLE...QSSQ...VD.S.IFSG  S. typh

LDGA--TLKDYDAKGVKGIETIKIQPNSLAAQRGLKSGDIIIGINRQMIENIR
IE..EMSN.GK.QGV.VNNVK.----GTP...I...K..V...A.Q.AVK..A
IE..EMSN.GQ.KGV.VSSVKA----..P...I...K..V...A.Q.PVK..A

                        ELNKVLETEPSAVALNILRGDSNFYLLVQ*  Hin47
                        ..R...DSK..VL....Q...--RH.P.N*  E. coli
                        ..R.I.DSK..VL....Q....SI...M.*  S. typh
```

FIG.4A.

```
TON  :                IVGGYKCEKNSQPWQVAVIN-----E---YLCGG VLID
PKAAB:                IIGGRECEKNSHPWQVAIYHY----SS---FQCGG VLVN
PIN  :                IVGGYTCGANIVPYQVSLN-----SGY---HFCGG SLIN
CHAA :                IVNGEEAVRGSWPWQVSLQDK---TGF---HFCGG SLIN
EST  :                VVGGTEAQRNSWPSQISLQYRSGSSWA---HTCGG TLIR
RP2A :                IIGGVESIPHSRPYMAHLDIV---TEKGLRVTCGG FLIS
SGT  :                VVGGTRAAQGEFPFMVRLSM-----------GCGG ALYA
SGBE :                ISGG----------DAIYSS----------TGRCSLGFNVRSGS
SGA  :                IAGG----------EAITTG----------GSRCSLGFNVSVNG
ALP  :               ANIVGG----------IEYSIN----------NASLCSVGFSVTRGA
hin47:               AEQFGG          RGESKR          N FRGLGSGVTINAS
                     ******                            **
con                        <------>  <------>  <------>
                                  # (His57)
TON  : -----PSWTTAAHCY--S-----N-NYQ-VLLGRNLFK-DEPFAQRRLV
PKAAB: -----PKWLTAAHCK--N-----DNYEV-WL-GRHNLFENENT-AQFFGV
PIN  : -----SQWVSAAHCY--X-----SGIQV-RL-GEDNINVVEGN-EQFISA
CHAA : -----ENWVTAAHCG--V-----TTSDV-VVAGEFDQGSSSEK-IQKLKI
EST  : -----QNWMTAAHCV--D-----RELTFRVVVGEHNLNQNNGT-EQYVGV
RP2A : -----RQFVLTAAHCK----------GREIT-VTLGAHDVRKREST-QQKIKV
SGT  : -----QDIVLTAAHCV--SGSGNTSIT-ATGGVVDL-QSG-A-AVKVRS
```

FIG.4B.

```
SGBE :     TYYFLTAGHCT--D-----GATT-WWA---------NS-ARTTVL
SGA  :     VAHALTAGHCT--------NISASW-----------------SI
ALP  :     TKGFVTAGHCGTVN----AT-AR-IG-------------GAVVG
Sal.T:     KGYVVINNHVVDNASVIKVQLSDGR
hin47:     KGYVLTNNHVIDEA    DK-IT-VQ-------------LQDGRE
             ********

con        <--------->        <----><->         <----


                        #(Asp102)
TON  : RQS-FRHPDYIPLI¦PVHDH--SNDLMLLHLSEPADITGGVKV-------
PKAAB: TAD-FPHPGFNLSAD-GKDY--SHDLMLLRLQSPAKITDAVKV-------
PIN  : SKS-IVHPSYN-----SNTL--NNDIMLIKLKSAASLNSRVAS-------
CHAA : AKV-FKNSKYN-----SLTI--NNDITLLKLSTAASFSQTVSA-------
EST  : QKI-VVHPYWN-----TDDVAAGYDIALLRLAQSVTLNSYVQL-------
RP2A : EKQ-IIHESYN-----SVPN--LHDIMLLKLEKKVELTPAVNV-------
SGT  : TKV-LQAPGYN-----G-T---GKDWALIKLAQPIN----QPT-------
SGBE : GTT-SGS-SF-----------PNNDYGIVRYTNTTIPX   DGTVG-------
SGA  : GTR-TGT-SF-----------PNNDYGIIRHSNPAAA    DGRVYLYNGS---
ALP  : -TFAARV--F-----------PGNDRAWVSLTSAQTL----LPRVANGSS
hin47:   FKAKLVG        KDEL   SDIALVQLEKPSNL  TEIKFADSDKLRVGDF
                               **********

con    ><---><-------->         <------><--->  <---
```

FIG.4C.

```
TON  : ---IDLPT--KEPKVGSTCLASGWGSTNPS-E-MVVSHDLQCVNIHLLSN
PKAAB: ---LELPT-QEPE-LGSTCEASGWGSIEPGPDDFEFPDEIQCVQLTLLQN
PTN  : ---ISLPT-SCAS-AGTQCLISGWGNTKSS--GTSYPDVLKCLKAPILSD
CHAA : ---VCLPSASDDFAAGTTCVTTGWGLTRY--¦-ANTPDRLQQASLPLLSN
EST  : ---GVLPRAGTILANNSPCYITGWGLTR-T--NGQLAQTLQQAYLPTVDY
RP2A : ---VPLPSPSDFIHPGAMCWAAGWGKTGVR--DPT-SYTLREVELRIMDE
SGT  : ---LKIAT--TTAYNQGTFTVAGWGANRE---GGSQQRYLLKANVPFVSD
SGBE : GQDITSAA   NATVGMAVTRRGSTT--------------GTHSGSVTAL
SGA  : YQDITTAG   NAFVGQAVQRSGSTT--------------GLRSGSVTGL
ALP  : FVIVRGST---EAAVGAAVCRSGRTT--------------GYQCGTITAK
hin47: TVAIGNPFGLGQTVTSGIVSALGRST               GSDSGTYENY
                          *********               ****
con         --->         <------<-->          <------------->

                                                 #(Ser195)
TON  : EKCIE--TYKDNVT-DVMLCA-G-E---MEGGK-DTCA--GDSGGPLIC-
PKAAB: TFCAD--AHPDKVT-ESMLCAGY-L---P--GGKDTCM--GDSGGPLIC-
PTN  : SSCKS--AYPGQIT-SNMFCAGY-L---E--GGKDSCQ--GDSGGPVVC-
CHAA : TNCKK--YWGTKIK-DAMICA-G-A---S--GV-SSCM--GDSGGPLVC-
EST  : AICSSSSYWGSTVK-NSMVCA-G-G---D--GVRSGCQ--GDSGGPLHC-
RP2A : KACVDYRYYEYKF----QVCV-GSP---T--TLRAAFM--GDSGGPLLC-
SGT  : AACRS--AYGNELVANEEICA-G-YPDTG--GV-DTCQ--GDSGGPMFR-
SGBE : NATVN--YGGGDVV-YGMIRT-N------------VCAEPGDSGGPLYS-
```

FIG.4D.

```
SGA   : NATVN--YGSSGIV-YGMIQT-N------------VCAQPGDSGGSLFA-
ALP   : NVTAN--Y-AEGAV-RGLTQG-N-A---------CMGR--GDSGGSWITS
hin47:                IQT D A          AVNR  GNSGGALVNLN
              *** * *        ****  ********
con                   <---->          <---->  <-><-----

TON   : D-----GVLQGITSGGA-TP----C-A-KP--K-T-PAIYAKLIKFT-SW
PKAAB: NG-----MWQGITSWGH-TP----C-GSA---N-K-PSIYTKLIFYL-DW
PTN   : SGK-----LQGIVSWGS--G----C-AQK---N-K-PGVYTKVCNYV-SW
CHAA  : KKN-GAWTLVGIVSWGS-ST----C-S-T---S-T-PGVYARVTALV-NW
EST   : LVN-GQYAVHGVTSFVSRLG----C-NVT---R-K-PTVFTRVSAYI-SW
RP2A  : --A-GV--AHGIVSYG----------HPD---A-KPPAIFTRVSTYV-PW
SGT   : KDNADEWIQVGIVSWGY--G----C-A-R---PGY-PGVYTEVSTFA-SA
SGBE  : G-----TRAIGLTSGGS-GN----C-S-S---G-G-TTFFQPVTEALVAY
SGA   : G-----STALGLTSGGS-GN----C-R-T---G-G-TTFYQPVTEALSAY
ALP   : A-----GQAQGVMSGGN-VQSNGNNCG-IPASQ-R-SSLFERLQPIL-SQ
hin47:        GELIGINTAII SP SGGNAG IAFAI P SNQASNLVQQIL
                   ????
con     >     <--------->                  <-----><------
```

FIG.4E.

```
TON  : IKKVMKENP
PKAAB: IDDTITENP
PIN  : IKQTIASN
CHAA : VQQTLAAN
EST  : INNVIASN
RP2A : INAVIN
SGT  : IASAARIL
SGBE : GVSVY
SGA  : GAIVL
ALP  : YGLSLVIG
hin47: EFGQVRRGLLGIIKG
con  ------->
```

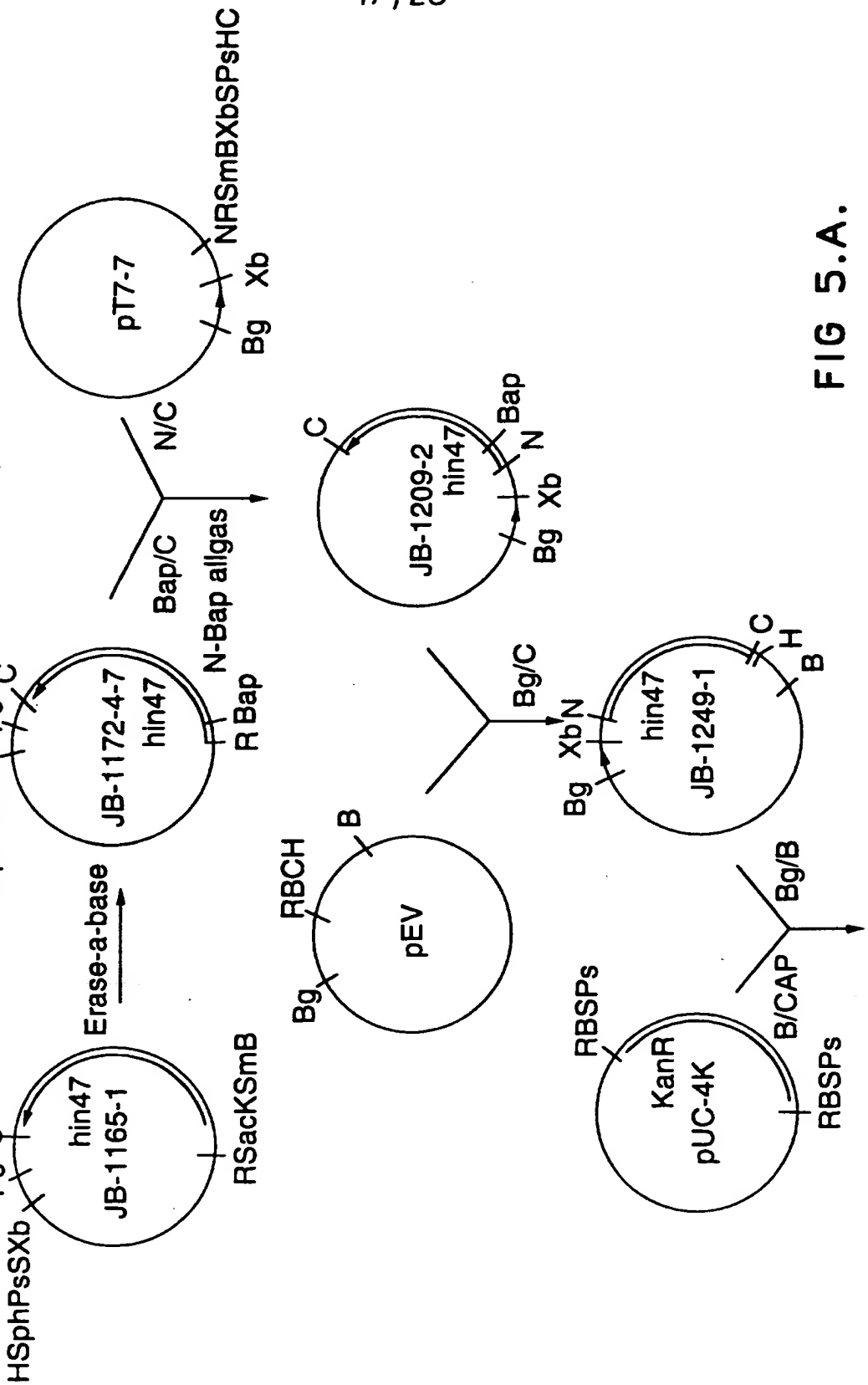

pT7-7
NRSmBXbSPsHC
Bg Xb
N/C
Bap/C
N-Bap allgas
JB-1209-2
hin47
C
Bap
N
Bg Xb
HSphPsSXb Ps C
JB-1172-4-7
hin47
R Bap
Erase-a-base
Bg/C
Bg XbN
hin47
JB-1249-1
C
H
B
HSphPsSXb
Ps C
hin47
JB-1165-1
RSacKSmB
RBCH
B
pEV
Bg
Bg/B
B/CAP
RBSPs
KanR
pUC-4K
RBSPs

FIG 5.A.

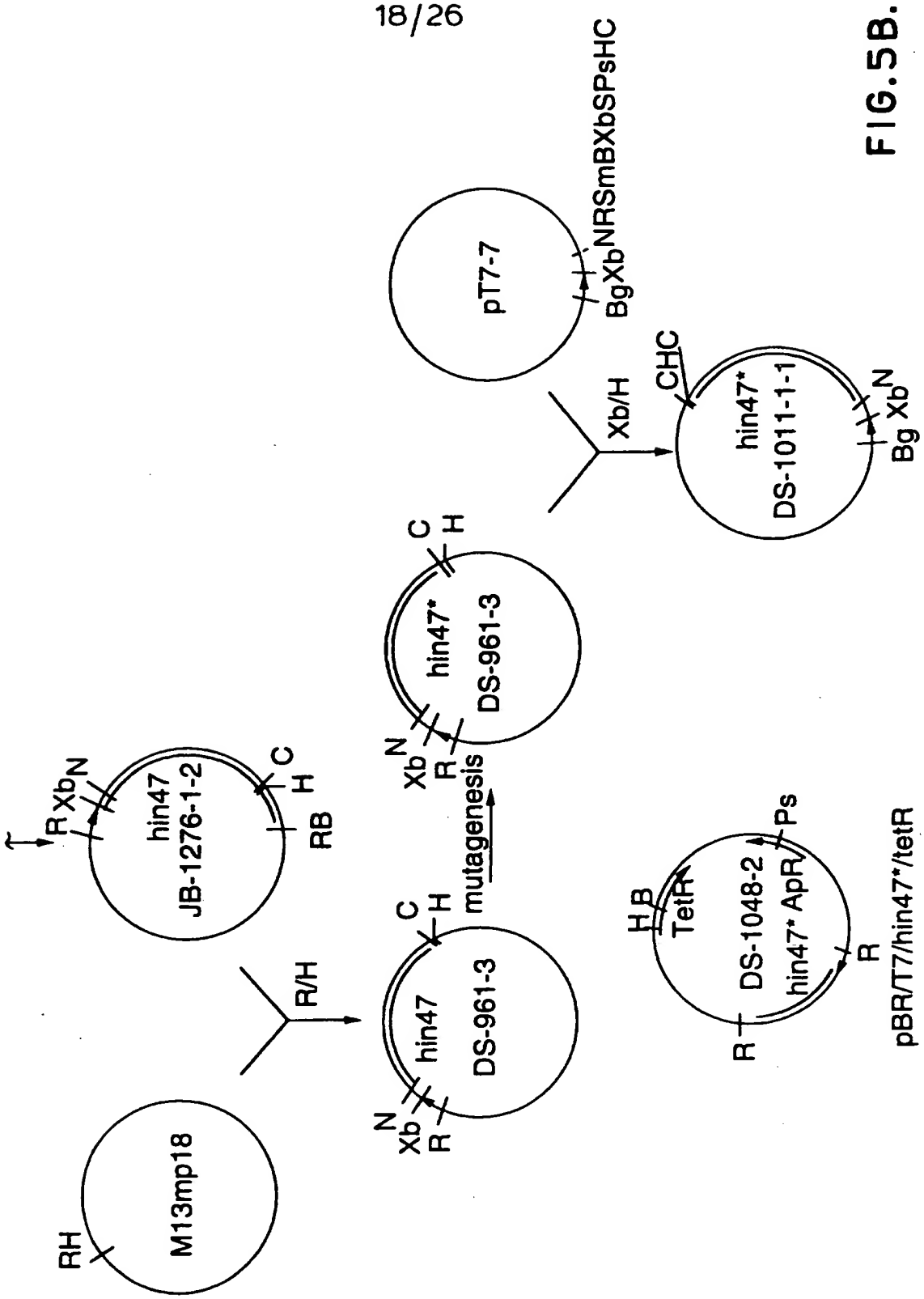

M13mp18
RH
R/H
hin47
DS-961-3
N
Xb
R
C
H
mutagenesis
hin47
JB-1276-1-2
R
Xb
N
C
H
RB
hin47*
DS-961-3
N
Xb
R
C
H
pT7-7
Bg
Xb
N
R
S
m
B
Xb
S
P
s
H
C
Xb/H
hin47*
DS-1011-1-1
CHC
Bg
Xb
N
H
B
TetR
DS-1048-2
hin47* ApR
Ps
R
R
pBR/T7/hin47*/tetR

FIG.5B.

PURIFACATION OF HIN47 MUTANT

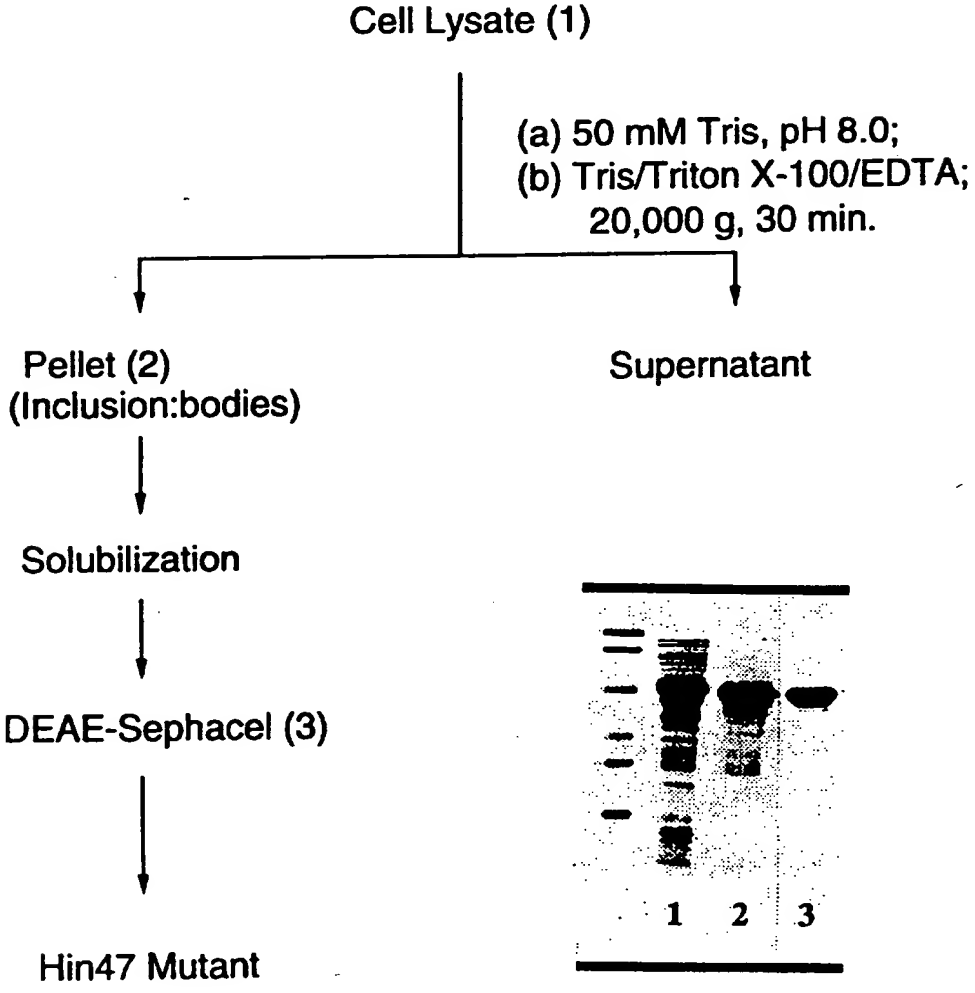
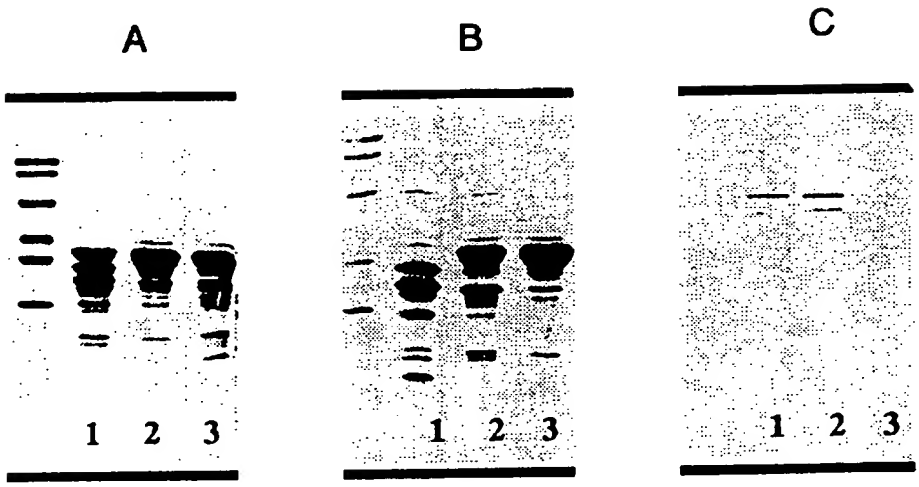


FIG.6.

Digestion of β-Casein by Hin47



1. β-Casein + Hin47
2. β-Casein + Mutant
3. β-Casein

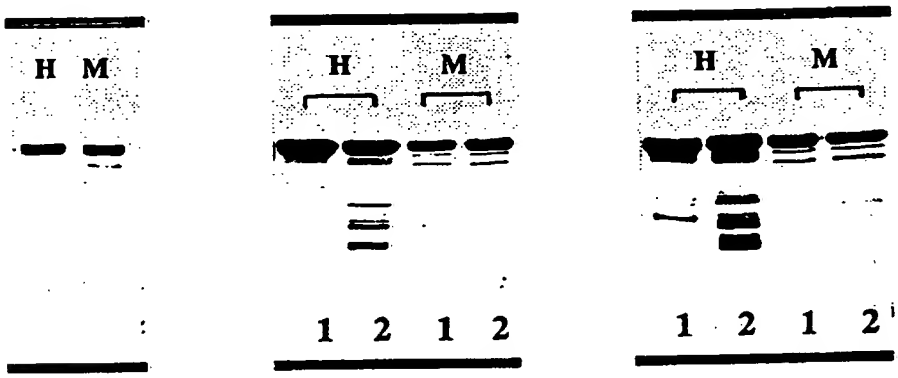
A. Each lane contains 5μg of β-casein, +/- 20 ng of Hin47 or mutant

B. Each lane contains 5μg of β-casein, +/-0.1 μg of Hin47 or mutant

C. Immuno-blot with rabbit anti-Hin47 antibody

FIG.7.

IMMUNO-BLOT ANALYSIS ON THE STABILITIES OF HIN47 AND MUTANT



Day 0 Day 10 Day 20

H: Hin47

M: Mutant

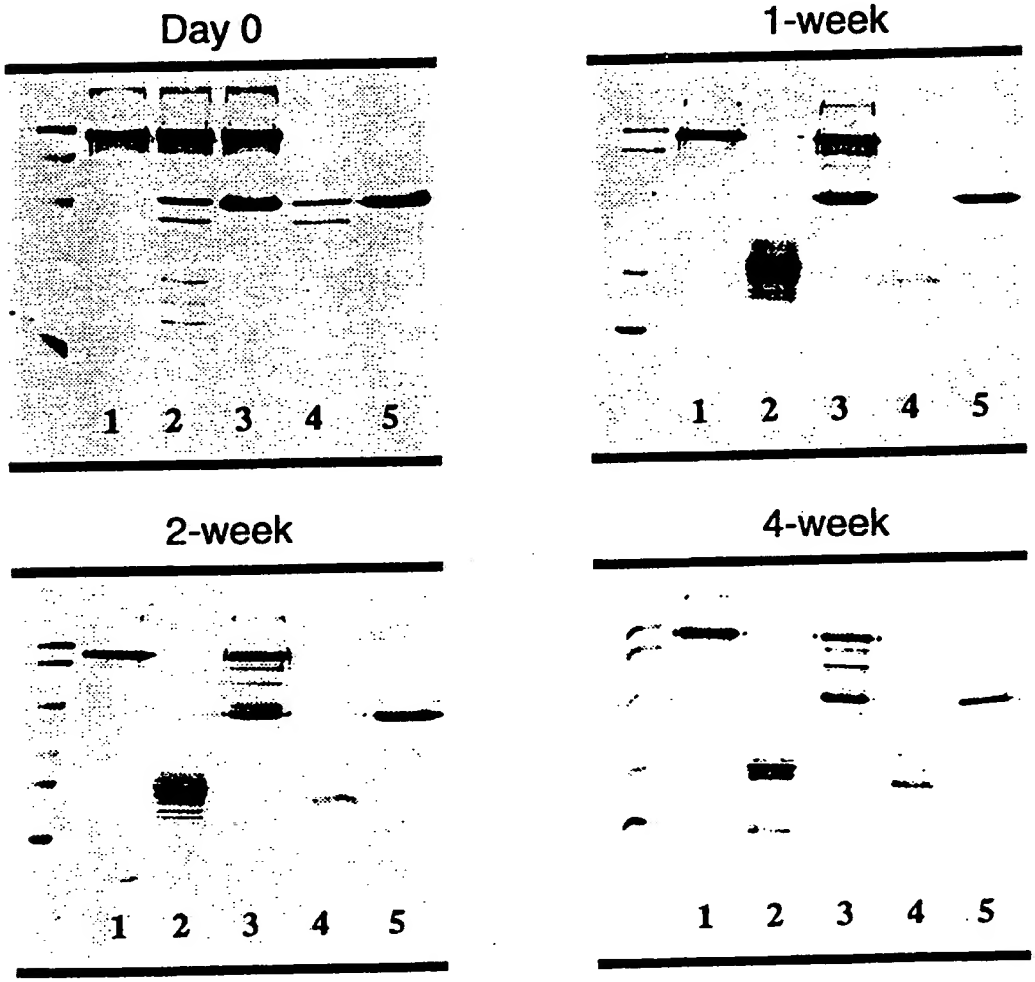
1. -20ºC

2. 4ºC

FIG.8.

22/26

Stability Studies on Mixed Antigens
in the Presence of Hin47 or Hin47 Mutant

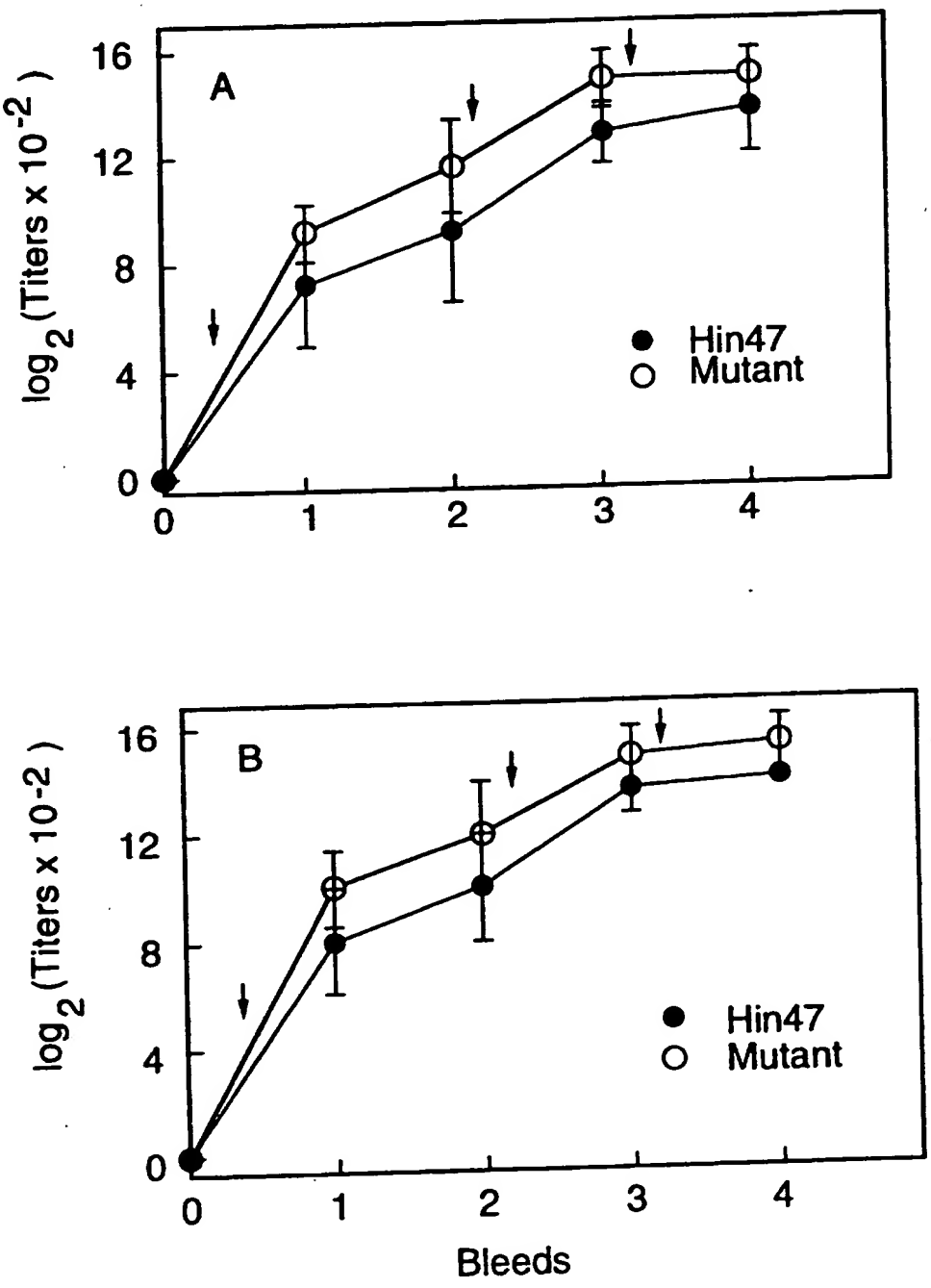


1. rTBP1
2. rTBP1 + Hin47
3. rTBP1 + Hin47 mutant
4. Hin47
5. Hin47 mutant

FIG.9.

SUBSTITUTE SHEET

Comparative Immunogenicity of Hin47 and Mutant in Mice



(A) Plat coating: Hin47 (B) Plate coating: mutant

FIG.10.

FIG.11A.

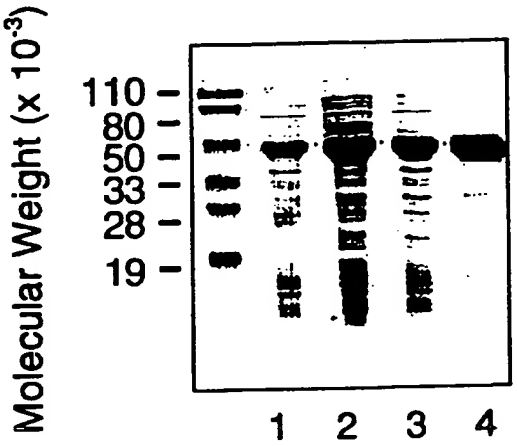
Comparison of Hin47

```
MKKTRFVLNSIALGLSVLSTSFVAQATLPSFVSEQNSLAPMLEKVQPAVV    SB33
                          M.....................    SB12

TLSVEGKAKVDSRSPFLDDIPEEFKFFFGDRFAEQFGGRGESKRNFRGLG    SB33
.................................................    SB12

SGVIINASKGYVLTNNHVIDEADKITVQLQDGREFKAKLVGKDELSDIAL    SB33
..................G.....................Q.....    SB12

VQLEKPSNLTEIKFADSDKLRVGDFTVAIGNPFGLGQTVTSGIVSALGRS    SB33
.................................................    SB12

TGSDSGTYENYIQTDAAVNRGNSGGALVNLNGELIGINTAIISPSGGNAG    SB33
.................................................    SB12
```

FIG.11B.

```
IAFAIPSNQASNLVQQILEFGQVRRGLLGIKGGELNADLAKAFNVSAQQG     SB33
.....................C.............................     SB12

AFVSEVLPKSAAEKAGLKAGDIITAMNGQKISSFAEIRAKIATTGAGKEI     SB33
.........................G.........................     SB12

SLTYLRDGKSHDVKMKLQADDSSQLSSKTELPALDGATLKDYDAKGVKGI     SB33
.....................G.............................     SB12

EITKIQPNSLAAQRGLKSGDIIIGINRQMIENIRELNKVLETEPSAVALN     SB33
.....................................K.........     SB12

ILRGDSNFYLLVQ*                                         SB33
....N........**                                        SB12
```

Purification of Hin47 Mutant H91A From *E. coli*



1. *E. coli* Whole cells
2. Soluble proteins after 50 mM Tris,pH 8, extraction
3. Flow-through fraction after DEAE Sephacel column
4. Purified H91A from hydroxyapatite column

FIG.12.